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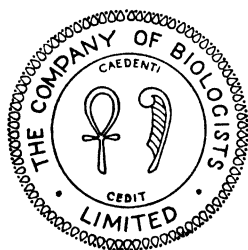
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# The Giant Nerve-fibres in the Central Nervous System of Myxicola (Polychaeta, Sabellidae)

BY

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**With fourteen Text-figures and three Plates**

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## I. INTRODUCTION

THE giant nerve-fibres which occur in the central nervous system of many Annelids are such large and conspicuous structures that they have aroused a considerable amount of interest and a large body of literature has grown up about them. During the latter part of the nineteenth century and the first years of this century, when the general morphology of the Annelida and the anatomy of the central nervous system of this phylum were being extensively investigated, there appeared frequent references to the occurrence of giant nerve-fibres in the Polychaeta. Most investigators were interested in classifying the species or in phylogenetic studies and merely recorded the presence or absence of giant fibres and giant nerve-cells. In many cases the size of the fibres was so great and they corresponded so little to the conventional picture of vertebrate peripheral nerve-fibres that their nature was in doubt for a long time and many hypotheses concerning their function were

postulated. For reviews of this controversial and nearly forgotten subject see Eisig, 1887; Friedländer, 1889; Lewis, 1898; Ashworth, 1909; and Stough, 1926. Many of these theories now seem fantastic, e.g. supporting functions and even excretory functions were suggested for the fibres. Such ideas were based entirely upon the appearance of the structures and in the absence of supporting experimental evidence each theory retained an element of probability. Friedländer (1889) suggested that the giant axons of Annelids were concerned with the quick end-to-end contraction ('startle-reaction') that earthworms and some Polychaetes exhibit when strongly stimulated. This theory received support from the experiments of Bovard (1918) who cut the nerve-cord of the earthworm and found a significant correspondence between the time of regeneration of the giant fibres and the return of quick contraction of the body, as contrasted with regeneration of the rest of the nerve-cord and the return of slower locomotory movements. Yolton (1923) was able to confirm this hypothesis by cutting the giant nerve-fibres without severing the rest of the nerve-cord; he found that the quick contractions failed to pass the site of injury. Subsequently, Eccles, Granit, and Young (1933), Rushton (1945, 1946), and Bullock (1945) have studied the action potentials of these fibres and have described their conduction properties.

Apart from the early detailed studies of the giant fibres of several different Polychaetes by Rohde (1887), Friedländer (1889), Gamble and Ashworth (1900), Ashworth (1909), and others, the majority of investigations have been confined to the Oligochaeta and recent physiological studies have been carried out exclusively on the earthworm. For this reason it was decided to examine the giant nerve-fibres of several Polychaetes with the aid of recently introduced histological methods. These giant fibres reach their greatest development in some sedentary Polychaetes (Spiomorpha and Sabellimorpha) and, since a number of early authors have commented on the extraordinarily large size of the giant axon of the Sabellid, *Myxicola infundibulum* Rénier, this species was selected for study.

## II. TERMINOLOGY

Knowledge of the nervous system of the Invertebrates has grown up with that of the Vertebrates and the resultant terminology is in part a borrowing from the Vertebrates and in part peculiar to the invertebrate forms; in either case it is frequently most inappropriate and misleading. It is proposed to use a consistent terminology in this paper for the features of the annelid central nervous system and the following terms have been adopted in preference to such ambiguous words as ventral chain, neurilemma, epineurium, and neurochords:

*Supra-oesophageal ganglia, oesophageal connectives, sub-oesophageal ganglion, nerve-cord, nerve-cord sheath*, and *peripheral nerves* refer to structures usually designated as such.

*Ganglia of the nerve-cord.* Swellings of the nerve-cord will be designated as ganglia only in those cases where there is definite evidence that they embody discrete accumulations of nerve-cells.

*Giant nerve-fibres* (or *axons*). Employment of these terms to designate the conspicuously large fibres of Annelids follows current English usage.

It is now necessary to define what is a giant nerve-fibre. First of all, it clearly refers to those nerve-fibres of any species which are disproportionately greater in size than the other nerve-fibres of the animal. They need not be great in absolute units, however. In the Enteropneusta, for example, they are $6\mu$ or less in diameter (Bullock, 1944). But their size must be strikingly greater than that of the other fibres of the individual. Secondly, a functional distinction can be drawn, since in all Invertebrates in which this aspect has been investigated it has been shown that the giant fibres are concerned with quick escape or withdrawal mechanisms effected by widespread and synchronous or nearly synchronous muscular contractions (Yolton, 1923; Stough, 1930; Young, 1938, 1939; Ten Cate, 1938; Bullock, 1945; Rushton, 1946). It may be necessary to modify this criterion as more is learnt of the functions of these structures in various groups, but the existing evidence is compatible with this viewpoint. Hanström (1928) has suggested that there probably exist all gradations between ordinary nerve-fibres and giant nerve-fibres and Young (1936) found a continuous spectrum of fibre diameters in the nerve to the stellate ganglion of the cuttlefish. In this case, however, the giant axons 'were segregated by themselves in a corner of the nerve', while in the stellar nerves of the same animal the giant fibres were much larger than any others in the nerve. There is seldom any difficulty in determining whether any given species possesses a discrete system of nerve-fibres which can be termed giant axons. The following definition then would appear to satisfy existing knowledge: giant fibres are those fibres which have a much greater size than the other nerve-fibres of the animal; they form an essential nervous component of a system which effects a rapid, widespread, and synchronous response.

### III. HISTORICAL REVIEW

#### 1. *Myxicola infundibulum* Rénier

Claparède (1861) was the first observer to record the presence of giant fibres in the nerve-cord of Annelids and in subsequent papers (1870, 1873) he gave a description of the nervous system and the giant fibres of *M. infundibulum*. According to this author the nerve-cord was double in the thorax, but one of the strands, after becoming enclosed within the sheath of the other, gradually tapered and disappeared; the other strand assumed a median position and continued posteriorly as the single nerve-cord. Ganglionic swellings were not pronounced and nerve-cells occurred throughout the length of the cord on the ventral and lateral surfaces. The giant fibre lay on the dorsal side of the nerve-cord (he regarded the giant fibre as being external to the nervous system) and had a diameter greater than that of the nerve-cord itself: $350\mu$ in a specimen 5 mm. in diameter. The fibre was usually cylindrical throughout its length and was invested by a sheath composed of numerous layers of nucleated supporting tissue which was continuous with the external

covering of the nerve-cord. Two fibres arose in the supra-oesophageal ganglia and one passed down each oesophageal connective, there attaining a diameter of $55\mu$. On reaching the nerve-cord the two fibres united, with consequent fusion of their axoplasm. He examined fresh as well as preserved material and noted that the axon contained a clear, homogeneous substance.

McIntosh (1877) confirmed the fact that the nerve-cord was double in the anterior third of the animal and that each strand contained a giant fibre in its dorsal region. Posteriorly the two strands united and the resulting single cord contained a large, dorsally situated axon. According to Pruvot (1885), the central nervous system consisted of supra-oesophageal ganglia divided into pairs of superior, inferior, and posterior lobes, oesophageal connectives joining the nerve-cord in setiger II and a nerve-cord double throughout its length. Two pairs of peripheral nerves arose from the nerve-cord in each segment. The giant axon divided into two longitudinal fibres in the thorax; anastomoses connected the two fibres together in the commissures uniting the halves of the nerve-cord in setiger II.

Meyer (1887, plates; 1888, text) has described the general anatomy of the nervous system in some detail. He found that the supra-oesophageal ganglia were provided with abundant nerve-cells; that they gave rise to two pairs of coronal nerves; and that the posterior lobes were very well developed. This author also considered that the nerve-cord was double throughout its extent, with two pairs of ganglia and two commissures in each segment. This bipartite arrangement, however, was obscured in the abdomen by the very large median fibre which overlay the nerve-cords and it was only in the thorax, where the median fibre divided into two lateral halves, that the true bipartite condition was revealed. Of the two thoracic giant fibres, one ended blindly and the other proceeded posteriorly as the median giant fibre of this region. The oesophageal connectives arose from the nerve-cord in the middle of setiger II. His illustration shows a longitudinal division of the nerve-cord and the giant fibre in segments III and IV, with commissures between segments II and III, and between III and IV.

A similar description of the giant fibre was given by Cunningham (1888) who observed that there were two fibres in the anterior region of the body, one of which terminated without joining the other, while the other fibre continued caudad as the median giant fibre. He also confirmed Claparède's observation that the giant fibres in the oesophageal connectives became continuous with one another in the lower part of the supra-oesophageal ganglia.

Friedländer (1889) disagreed with Meyer's observations and stated that the two anterior giant fibres definitely fused with one another in the thoracic region. He showed, in addition, that the giant fibre contained an apparently homogeneous mass; that its sheath was relatively and absolutely much thinner than that of Lumbricus and Mastobranchus (Oligochaeta and Polychaeta, respectively, with strongly myelinated giant nerve-fibres); and that the sheath blackened only slightly during treatment with osmium tetroxide. He looked for, but could not find, the nerve-cells of the giant fibre.

Wawrzik's study (1892) of the giant fibre of this species was incidental to his investigation of the nature of fibrillae, a study which led him to believe that neurofibrils and connective tissue fibrils were continuous with one another and of the same nature. He described fibrils passing out of the giant fibre into its fibrous sheath. It is possible that he actually saw nervous processes connected with the giant fibre and interpreted them as connective tissue fibrils. His photomicrograph of a transverse section shows the giant fibre badly distorted and shrunken from its sheath, except at a ventro-lateral corner. Now it is in this latter region that large peripheral branches arise from the giant fibre. He apparently observed such a branch and interpreted it in support of his hypothesis.

The erroneous descriptions of Claparède and Meyer, in placing the giant fibre outside the nerve-cord, can be attributed to uncertainty concerning the functional significance of this structure. It is difficult, however, to understand the reasons which led Meyer and Pruvot to conclude that the nerve-cord of this species was double throughout its length. They were probably influenced by the prevalent concept that the nerve-cords of all Annelids consisted of a ganglionic chain and they interpreted the longitudinal fibrous tracts of the cord as separate halves of the nerve-cord. It is certain, however, that double ganglia, transverse commissures, and a ladder-like structure simply do not exist as constant metameric characteristics in this species. All authors agreed, however, that the nerve-cord bifurcated anteriorly. Slight differences exist in the pictures given by Pruvot and Meyer, but both investigators have shown a double nerve-cord anterior to setiger IV, with two transverse commissures somewhere in setigers II and III. Friedländer (1889) has shown that the two thoracic giant axons actually fused together, contrary to the descriptions of Meyer and Cunningham. Although there is a certain amount of variation in the course of this fibre, and it is possible that the rare individual might actually show discontinuity of fibres in this region, a more likely explanation is that the latter two authors had based their descriptions on an incomplete series of sections, or on poorly fixed material. Another factor which may have led them into error is the fact that occasionally one of the longitudinal divisions of the nerve-cord in the anterior thorax is much smaller than the other and lacks a giant fibre. Isolated sections from such a region could provide a basis for the interpretations of these authors.

The following description of the central nervous system of *M. infundibulum*, therefore, can be compiled from the several accounts:

1. The supra-oesophageal ganglia lie in the prostomium and consist of a pair of superior and a pair of inferior ganglia from which arise prostomial and coronary nerves, and a pair of posterior lobes.

2. Oesophageal connectives join the nerve-cord in setiger II.

3. The nerve-cord is single throughout most of its length but is double in part of the first four thoracic segments where two commissures unite the two halves of the cord.

4. A giant fibre of exceptionally large size extends throughout the central nervous system. It arises in the supra-oesophageal ganglia as two small fibres, one of which extends down each oesophageal connective. The two fibres fuse with one another in the sub-oesophageal ganglion. and the resultant fibre extends to the posterior end, dividing where the cord divides, and fusing where the cord is single. It possesses a distinct sheath.

In a short preliminary note to the present paper, Nicol and Young (1946) have confirmed the description given above of the giant fibre of *Myxicola infundibulum*, and have shown in addition that the sheath of the fibre was negative towards osmium tetroxide and did not possess a pronounced myelin sheath. They found that the giant fibre was a syncytial structure, connected with small nerve-cells throughout its length, and they substantiated this fact by the observation that the giant fibre did not degenerate after section during periods up to 16 days following injury. They observed, moreover, that the fibre gave off branches to the peripheral nerves along its entire course.

Several authors have stated that this species gives a very vigorous and quick contraction when mechanically stimulated (Montagu, 1808; Friedländer, 1889). Claparède (1870) found that during this contraction the animal shortened its length by half. Since it has been proved definitely that the giant fibres of earthworms are concerned with the transmission of impulses concerned with the quick end-to-end contractions of the body, there is little doubt that the giant axon of *M. infundibulum* also forms part of the nervous pathway for the vigorous contractions of this species.

## 2. *M. aesthetica* Claparède

*M. aesthetica* is a small replica of the larger species, *M. infundibulum*. The internal anatomy of this species is largely unknown. De St. Joseph (1894) has given several details concerning the blood vascular system, but the nervous system has never been described. Okada (1932, 1934), in an extensive and dexterous investigation, has shown that this animal possesses remarkable powers of regeneration. Any body segment could give rise to a new head anteriorly and a new tail posteriorly, and several heads could be induced to grow on the same animal by cutting the nerve-cord. He considered that the nerve-cord was necessary for regeneration to proceed to completion, but he did not concern himself with the structure and growth of the internal organs.

It is proposed in this paper to give a description of the main features of the central nervous system of *Myxicola infundibulum* and *M. aesthetica*, in so far as they are related to the arrangement of the giant nerve-fibres of these species. The structure of the giant axons has been investigated with the following points in view, in order to establish an anatomical background for the study of behaviour and nervous function of the Sabellidae: (1) size and course of the giant axons; (2) sheaths; (3) nerve-cell bodies; (4) syncytial or

unicellular nature; (5) branches or efferent connexions. It is hoped to present further data concerning the functioning of these structures in a subsequent communication.

## IV. Material and Methods

The specimens of Myxicola used in this investigation were obtained from the Plymouth Laboratory of the Marine Biological Association. *Myxicola infundibulum* is a tubicolous form dwelling in sand–clay flats near low spring-tide mark. Montagu (1808), who first discovered this species on the Saltstone near Salcombe, Devon, has given a good description of its occurrence there. It is distributed fairly abundantly over a short stretch of the shore, and the burrows of the animals may be readily detected by the slight protrusion of the blackish gelatinous tube which projects above the surface. The animal lives in a long and very voluminous mucous tube which fills its burrow. Mature specimens are about 10 cm. to 13 cm. long when normally extended, and about 6 mm. wide. The mean number of segments lies near 130. There are 8 setigerous segments in the thorax which show the usual Sabellid characteristics. It lives well in the laboratory for several months when kept in running sea-water. A group of animals usually forms a thick mucous mass which tends to float at the surface, but this difficulty can be overcome, however, by supplying the animal with sand in which to burrow.

*M. aesthetica* resembles, in miniature, the larger species, *M. infundibulum*. Specimens were obtained in dredgings from Asia shoal, particularly in association with Ascidiella. It is tubicolous, secreting a hyaline, jelly-like tube. Larger specimens are about 15 mm. long and 1 mm. wide and have up to 50 segments. The thorax consists of 3 setigerous segments. This species is very hardy and will live for long periods in finger-bowls containing sea-water.

The nervous elements of the annelid nervous system stain poorly with routine stains and require special techniques in order to render the individual neurones distinguishable. Since methods of silver impregnation are usually necessary, the fixatives which can be employed are limited by this factor as well as by the necessity of obtaining adequate preservation of histological and cytological detail. The several techniques which were tried are discussed as follows.

### 1. *Fixatives*

The necessity of obtaining good fixation is well emphasized by the errors which have crept into the work of several earlier investigators who have drawn conclusions regarding normal structure from patent artifacts. Rohde (1887), for example, has described nerve-fibres traversing large spaces which he found between the giant axon and its sheath in certain Polychaetes, spaces obviously due to shrinkage of the axoplasm during fixation and subsequent treatment. A number of investigators have stated that the fixative should be isotonic, as regards its neutral salt content, with the tissue studied in order to obtain optimal preservation (Carleton, 1922; Young, 1935; Cole, 1946; and others). With the exception of special cases where other factors were involved, all

fixative solutions employed in this study were made isotonic with sea-water, either by making up the solution in sea-water itself or by the addition of a suitable quantity of some indifferent salt.

A series of commonly used fixatives was tried out on *M. infundibulum* in an attempt to discover one which would give both reasonably good fixation and permit subsequent treatment with silver solutions. The two solutions which were found to be most satisfactory, in consequence, were Bouin's fluid and picro-formol, both of which were made up in sea-water. Picro-formol consisted of a saturated solution of picric acid in sea-water, 90 parts, and commercial formalin, 10 parts. These two reagents gave good fixation of the nervous system and the giant fibre, and it was possible to impregnate the neurones with silver after removal of the picric acid in 70 per cent. alcohol. A series of specimens of several species of Sabellids was fixed in chilled Bouin's fluid as recommended by Bodian (1937*a*), but no significantly better results were obtained by this treatment than by using the fixative at room temperature.

Simple solutions of formalin and picric acid caused considerable distortion of the giant fibre of *M. infundibulum* when paraffin embedding was employed. Young (1939) found formol and picro-formol solutions successful for preserving Cephalopod giant fibres, and Holmes (1942) obtained good results on prawn fibres with picric acid. Since neither formol nor picric acid alone permitted satisfactory silver impregnation in Myxicola, however, they were discarded. Flemming (without acetic) was also found to cause considerable shrinkage of the axon.

Helly's and Zenker's fluids, and post-chroming after formol-fixation, were also discovered to be good fixatives for the central nervous system. Mallory's triple and Heidenhain-azan stains gave satisfactory results after fixation in these media. These fixatives, however, greatly inhibited effective silver impregnation, properties already well known for the vertebrate central nervous system (Foley *et al.*, 1936; Bodian, 1937, 1937*a*).

Osmium tetroxide, in solutions of strength 0·2 to 1 per cent., was used for detecting the presence of myelin sheaths. Preservation was poor, however, and better results were obtained by employing the mixture of osmium tetroxide and picric acid recommended by Holmes (1942).

## 2. *Embedding media*

In an effort to eliminate some of the distortion caused to such a large, homogeneous mass of protoplasm as the giant nerve-fibres of the Polychaeta by paraffin embedding, some of the material was cut in Péterfi's celloidin-paraffin and in celloidin. Double embedding in methyl benzoate-celloidin gave very good results and recourse was had to celloidin sections for checking critical points only.

## 3. *Staining*

Previous workers who have investigated the annelid nervous system have found that the nerve-cells and their processes are difficult to stain and it is

only in exceptional circumstances, e.g. in the case of the giant nerve-cells of Lumbriconereids (Fedorow, 1928) where peculiarities of size facilitate identification, that giant axon neurones can be distinguished after routine methods. Earlier workers tried to reveal the nervous elements in the annelid nervous system by metallic impregnation, either with $OsO_4$ (Friedländer, 1888, 1894), or by the chrome-silver of Golgi (Nansen, 1887; Retzius, 1892), or silver methods of Cajal (Ramón y Cajal, 1904; Boule, 1908, 1909; Kowalski, 1909). *Intra vitam* methylene blue has been used with variable results. Retzius (1891) and Hamaker (1898) employed it successfully on Nereis and Krawany (1905) on the earthworm, but in general the method gave incomplete or negative results on Polychaetes (Retzius, loc. cit.; Evenkamp, 1931). More recently, methods of impregnating sections with silver have given good results among Invertebrates (Bodian, 1937; Bullock, 1944, 1945, 1945a; Holmes, 1942).

Two silver methods have been used extensively in this study, viz. impregnation of sections by activated protargol, and by Holmes's buffered silver nitrate-pyridine solutions. As might be expected, these gave different results in different species of Polychaetes and required to be modified in each case for the optimal effect. Impregnation was most satisfactory after fixation in picro-formol or Bouin's solutions, but a small degree of silver impregnation was also obtained in Helly-fixed material after subjecting the sections to 5 per cent. sodium bisulphite for 24 hours to remove the chrome salts, as recommended by Bodian (1937a). Counter-staining with Heidenhain-azan in this case gave a more complete picture. Nuclei, processes, giant fibres, and cytoplasm were all darkened with silver, and usually in that order of intensity, the smaller processes more intensely than the giant fibres.

Activated protargol was used in the manner recommended by Bodian (1936, 1937), employing Bayer's protargol (silver proteinate). The writer is indebted to Dr. William Holmes for assistance in using his method of silver impregnation which has since been published (Holmes, 1947). The basic method employed is as follows:

1. Place sections in 20 per cent. $AgNO_3$, in the dark, for $1\frac{1}{2}$ to 2 hours.
2. Wash, 10 min., in aqua dest., 3 changes.
3. Incubate, about 24 hrs., at 37° C., in a medium containing:
   Boric acid, 12·4 gm. per l. . . . . 55 c.cm.
   Sodium borate, 19 gm. per l. . . . 45 c.cm.
   Pyridine, 20 per cent. aqueous solution . . 5 c.cm.
   Silver nitrate, 1 per cent. . . . . 2·5 c.cm.
   Aqua dest. . . . . . . 392·5 c.cm.
   Strength of $AgNO_3$, 1/20,000
   pH of buffer mixture, 8·4
4. Wash, aqua dest., 3 min.
5. Reduce, 3 min., in
   Hydroquinone . . . . . . 1 gm.
   Sodium sulphite, 20 per cent. . . . 100 c.cm.
6. Wash, tap-water, 3 min.
7. Rinse, aqua dest.
8. Tone, gold chloride, 0·2 per cent., 3 min.
9. Rinse, aqua dest.

10. Place in oxalic acid, 2 per cent., until sections become purple.
11. Two rinses, in aqua dest.
12. Fix in sodium thiosulphate, 5 per cent., 5 min.
13. Wash, tap-water, 10 min.

The sections are then ready for counterstaining or mounting.

The following paragraphs refer to the effects of these reagents on the two species.

*Myxicola infundibulum.* Reasonably good impregnation, was obtained by both protargol and the basic buffered solutions and the results were similar in both cases. A deeper degree of impregnation resulted from increasing the silver strength of the buffer solution to 1/10,000.

*M. aesthetica.* The giant fibres and the nerve-cell nuclei were darkened by the basic buffered solution, but the cytoplasm of the nerve-cells and their processes were refractory to impregnation, even when the other tissues absorbed the silver heavily. Counterstaining with Heidenhain-azan revealed additional features. In the latter case it was found advisable to carry through a slide of *M. infundibulum* simultaneously in order to control the staining, since sections of *M. aesthetica* are very small and it is difficult to observe the differentiation of the stain under low-power objectives.

### 4. *Special methods*

Several methods were employed to investigate the problem of myelination. Besides fixation in osmium tetroxide, a method of greater morphological value than histochemical accuracy (Owens and Bensley, 1929; Lison, 1936; Dempsey and Wislocki, 1946; Baker, 1944), material was treated with Sudan IV and Sudan black and carried through Baker's test for lipines. Baker has described his method fully (1946) and his directions were followed carefully. In the case of sections to be treated with Sudan dyes, small pieces of the animal were fixed in Baker's formol-calcium and stored in formol-calcium-cadmium (Baker, 1944). After embedding in gelatine, the tissue was cut on the freezing microtome, mounted on slides, and treated with saturated solutions of Sudan black and Sudan IV in the manner recommended by Pantin (1946).

### V. OBSERVATIONS

#### 1. *Myxicola infundibulum* Rénier

##### (a) *Description of the central nervous system*

The central nervous system conforms to the typical annelid pattern and consists of supra-oesophageal ganglia, oesophageal connectives, sub-oesophageal ganglion, and a nerve-cord lying in the ventral body-wall (Text-fig. 1A).

The supra-oesophageal ganglia lie above the gut in the prostomium, and extend caudally into setiger I. They may be divided into a pair of inferior ganglia and a pair of superior ganglia (Text-figs. 2A and 2B). These are connected with one another by a median bridge of nervous tissue. The ventral, posterior regions of the inferior ganglia pass gradually into the oesophageal

giant fibre cell

supraoesophageal ganglia

giant fibre

oesophageal connectives

division of nerve cord

anterior thoracic nerve cord

anterior abdominal nerve cord

giant fibre

peripheral branch

posterior abdominal nerve cord
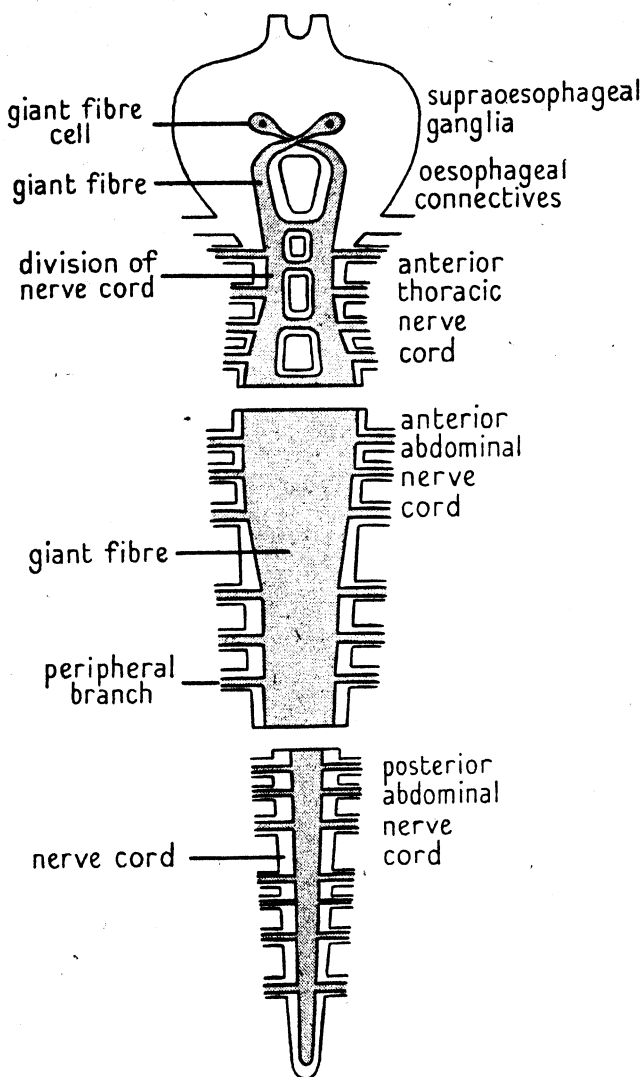
nerve cord

TEXT-FIG. IA. Diagram of the central nervous system and giant fibre of *Myxicola infundibulum* as seen from above. The nerve-cord is shown divided in three places in the thoracic region.

connectives which curve obliquely ventrally and posteriorly, and unite with the sub-oesophageal ganglion in setiger II (Text-figs. 3A and 3B). The general configuration of the supra-oesophageal ganglia is that of a short, broad mass, whose superior ganglia form a relatively high saddle embracing the excretory
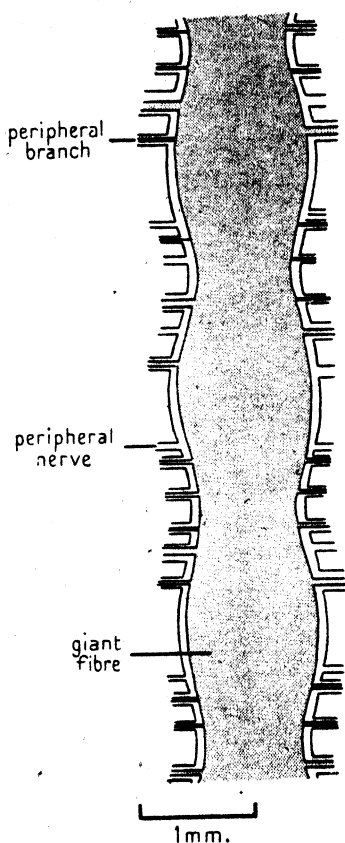


TEXT-FIG. 1B. Reconstruction of the nerve-cord and giant fibre in three anterior abdominal segments. Dorsal view.

duct of the large anterior nephridia. In a mature animal they are about 1·5 mm. broad, 0·5 mm. long, and 1 mm. high.

The nerve-cord for purposes of description may be divided into two regions: the thoracic nerve-cord and the abdominal nerve-cord. In the thorax the cord divides into two at several levels; in the abdomen it is usually single. The sub-oesophageal ganglion, lying in setiger II, is a relatively broad mass, larger than the rest of the nerve-cord. Behind this ganglion, in setigers II to IV, inclusive, the nerve-cord divides into two portions on three or four occasions. These divisions are fairly constant, but do not correspond exactly

in position, extent, and symmetry in different individuals (Text-figs. 1A and 4A). The following figures are typical and have been worked out in detail from serial sections of a mature animal. The first double region is about
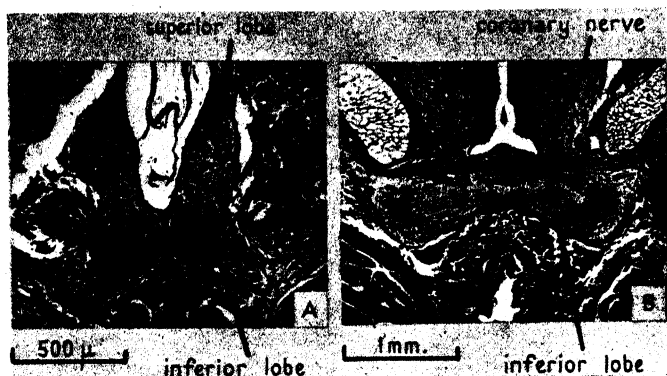


TEXT-FIG. 2A. Photograph of a transverse section through the supra-oesophageal ganglia of *M. infundibulum*. Picro-formol, cedarwood oil, paraffin, Holmes's silver, safranin.
TEXT-FIG. 2B. A horizontal longitudinal section through the same structure. Helly's, cedarwood oil, paraffin, Holmes's silver, Heidenhain-azan.
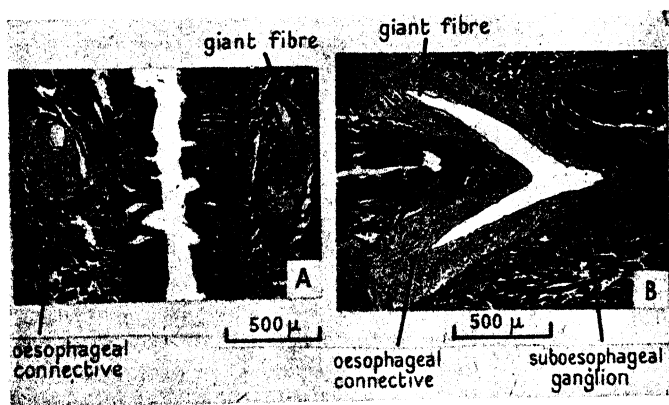


TEXT-FIG. 3A. Photograph of a horizontal longitudinal section through the oesophageal connectives of *M. infundibulum*. Helly's, cedarwood oil, paraffin, Holmes's silver, Heidenhain-azan.
TEXT-FIG. 3B. The same specimen. A section more ventrally through the sub-oesophageal ganglion.

$50\mu$ long and lies immediately behind the sub-oesophageal ganglion. The following two divisions are longer, about $100\mu$ to $150\mu$ in extent, and occur one per segment up to and including setiger IV. These separations of the cord show considerable variation in that they may be central and thus divide the cord into two equal portions; or they may lie laterally, giving rise to a

small lateral strand of nerve-cord which appears like an appendage of the main strand. Behind setiger IV, the cord remains single with the exception that irregularly, here and there, over stretches of less than 1 mm., a small, ventro-lateral strand of the nerve-cord may become cut off from the main body of the nerve-cord, only to rejoin it again.

This irregularity in pattern explains some of the discrepancies in the descriptions of Claparède, Pruvot, Meyer, and Friedländer, discussed previously. It is obvious that the bifurcations of the nerve-cord are not constant in number or in position. The features that they have in common are these: they are more extensive in the anterior thoracic segments than farther posteriorly; and they cause the nerve-cord, in the first four thoracic segments, to assume a bipartite condition with transverse commissures in each segment. The most satisfactory explanation of this condition would be given by knowledge of the development of the nerve-cord, but unfortunately the development of Myxicola is unknown. It may be suggested, tentatively, that the nerve-cord arises, as in other Polychaetes, from bilateral Anlagen in each segment, and that the adult condition is the result of a gradient in the degree of fusion of these embryonic neural masses, a fusion which proceeds farther in the abdomen than in the thorax. It is interesting to note, in this connexion, that the nerve-cord and its contained giant fibre frequently appear divided in the posterior few segments where it would be expected that increment in length occurs. At least in the related species, *Branchiomma vesiculosum* Montagu, Wilson (1936) has stated that new segments are laid down immediately in front of the pygidium after metamorphosis.

Meyer (1888) and Pruvot (1885) have described two pairs of nerves per segment. Careful reconstructions of serial sections show, however, that the true condition is more complicated. The number of peripheral nerves in each segment is not constant. There are actually five to seven nerves arising from each side of the nerve-cord in each metamere and these are of variable size, some containing one or a few fibres, others a large number. The mean number is six pairs and they are grouped into two fields. There is an anterior set of about three nerves on each side, occurring from the level of the intersegmental septum into the anterior third of the segment. Separated from this set by a considerable interval is a posterior set of another three nerves lying in the posterior third of the segment, in front of the posterior septum of that metamere (Text-fig. 1B). This may be compared with the condition which obtains in the Maldanids studied by Lewis (1898), but in these forms the arrangement was even more irregular and no pattern could be discerned. The peripheral nerves arise from the ventro-lateral border of the nerve-cord and proceed dorsally, between the circular and longitudinal muscle layers, towards the dorso-medial surface of the animal (Text-fig. 11; Pls. 1, 2 and 3, figs. 1, 12, 13, and 18). Besides these nerves, groups of fibres and single fibres leave the nerve-cord either ventrally to enter the sub-epidermal tissue, or laterally to innervate the longitudinal muscles lying immediately lateral

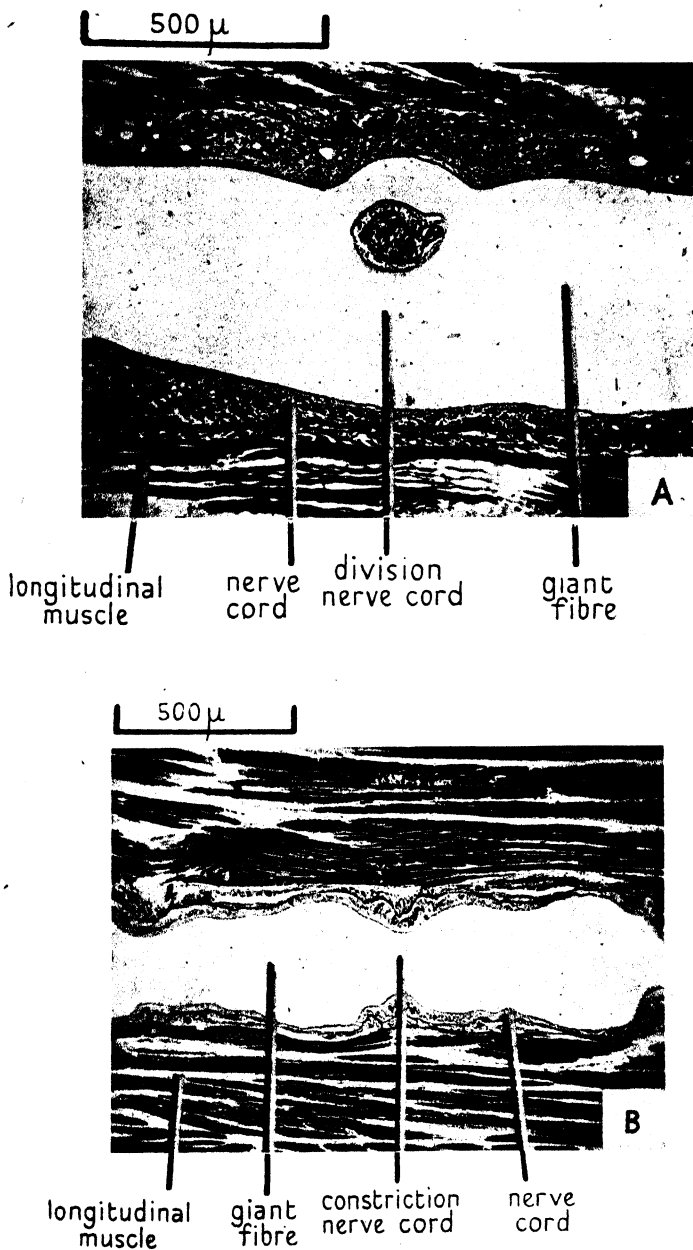TEXT-FIG. 4. Horizontal longitudinal sections through the thoracic nerve-cord of *M. in-fundibulum*. Helly's, cedarwood oil, paraffin, Holmes's silver, Heidenhain-azan. Text-fig. 4A is from a region lying slightly more anterior to that shown in 4B.
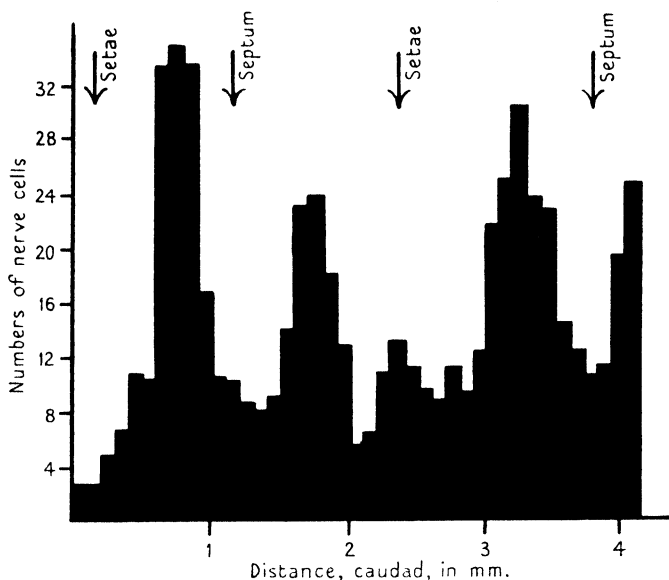
to the nerve-cord, or the circular muscles occurring in the intersegmental septa.

Several features of the nerve-cord of this animal can be observed in the living state by careful dissection. Because the animal contracts so violently it is necessary to narcotize it as a preliminary: immersion in 5 per cent. ethyl alcohol in sea-water for 15 minutes is effective. It is then slit open along the dorsal mid-line, which can be readily identified from the position of the ventral faecal groove, and the intersegmental septa are transected on either side of the gut. The animal is pinned out in a dissection dish and the gut stripped off with bent forceps under a binocular microscope. The nerve-cord is revealed by the position of the ventral blood-vessel which clings to its superior margin; it lies flush with the peritoneal surface of the longitudinal musculature. Two facts are then apparent. First of all, the nerve-cord is swollen in each segment, between the levels of the anterior and posterior septa, forming a beaded strand; secondly, it contains a large, clear, hyaline tube, the giant fibre, which is similarly expanded in each segment, forming a series of segmental swellings. In an attempt to determine the nature of these segmental swellings, the nerve-cords of several specimens have been reconstructed by plotting the horizontal plane of greatest diameter in transverse serial sections. Text-fig. 1B represents the result of such a reconstruction in the region of three anterior abdominal segments of a mature animal killed and fixed in a semi-contracted condition. The nerve-cord here shows constrictions at septal levels and swells out in the middle of each segment, the diameters alternating from about 1·3 mm. to 1 mm. It will be noticed, in addition, that the giant fibre shows the same alternations in diameter and follows closely the variations in cord volume (Text-fig. 4B).

Before considering, however, whether such swellings can be designated correctly as ganglia, it is necessary to consider their nerve-cell content. The usual significance of the term ganglion is that of an accumulation or aggregation of nerve-cells (Maximov and Bloom, 1944, p. 216). Nerve-cells are abundant in the supra-oesophageal ganglia (cf. Meyer, 1888; Johansson, 1927) and in the sub-oesophageal ganglion, and occur throughout the length of the nerve-cord. An enumeration of the total number of nerve-cells in several segments was made by counting all the nerve-cell nucleoli in a series of transverse serial sections, and the results are shown in Text-fig. 5. The nerve-cells reach their greatest density in the posterior region of the nerve-cord of each segment, in front of the intersegmental constriction, decrease in numbers at the septal level, and show a secondary and lesser accumulation in the anterior portion of each segment at the level of the setae. Accumulations of nerve-cells are thus apparent in the anterior and posterior portions of each segment, and these correspond, respectively, with the anterior and posterior grouping of the peripheral nerves. On the other hand, they are not directly correlated with the segmental swellings of the nerve-cord, most of which can be related to the bulging of the giant axon in the centre of each segment. This pattern possibly corresponds to the arrangement which occurs

in other Sabellids (e.g. Sabella, Thomas, 1940; Laonome, Evenkamp, 1931) where there are a pair of anterior and a pair of posterior ganglia per segment, but it is so much less clearly marked that it does not warrant the special designation of ganglia.

The nerve-cord lies in a ventral cleft within the longitudinal muscle layer and it is separated from the latter by a narrow coelomic space which descends



TEXT-FIG. 5. Histogram showing the distribution of nerve-cells at successive levels in the nerve-cord. The middle of each segment (setae) and the intersegmental level (septum) are marked by arrows.

almost to the level of the circular muscles (Text-fig. 11; Pl. 1, figs. 1, 5; Pl. 3, fig. 13), except at intersegmental levels where the septum bridges the gap between the nerve-cord and the body-wall. It is attached above by a ventral, longitudinal mesentery throughout its length, which contains chloragogen cells and the ventral blood-vessel. The nerve-cord is surrounded externally by a sheath consisting of three components. Externally lies a layer of flattened epithelial cells. Beneath this peritoneum there is a thin sheet of connective tissue enclosing a layer of circularly and longitudinally arranged muscle-fibres and small blood-vessels. This layer expands below, where it contains a relatively large amount of circular muscle-fibres which are continuous with those of the body-wall. It fuses with the septa at intersegmental levels and the enclosed muscle-fibres extend out as a broad sheet within the septa. Within this layer is the tunica propria of the nerve-cord. This consists of a compact band of connective tissue completely investing the whole cord with the exception of the places where the peripheral nerves emerge. It

forms a distinct sheath, sharply marked off from the other layers about the nerve-cord. It is composed rather of a sheet of tissue than of separate fibres and is probably collagenous since it shows a marked affinity for the aniline blue and acid fuchsin of Mallory's triple and van Gieson's connective tissue stains, respectively. Sections treated with Taenzer-Unna's orceïn for elastin failed to show any elastic tissue fibres.

The nerve-cord contains four separable components: the nerve-cells and their processes; the neuropile; the giant fibre and its sheath and branches; the supporting elements. In a cross-section it is seen that the giant fibre occupies a relatively large volume of the nerve-cord and lies dorsally to the nerve-cells and neuropile (Text-fig. 11; Pl. 1, figs. 1 and 5; Pl. 3, fig. 13). Laterally and superiorly it is situated close to the sheath of the nerve-cord from which it is separated by a layer of fine fibres and its own fibrous and cellular sheath. The nerve-cells lie ventrally and laterally in the cord, decrease in numbers dorsally, and in the upper half of the cord they are very sparse, only one or two occurring here and there in the narrow space between the giant fibre and the sheath of the nerve-cord (Pl. 1, fig. 1). The neuropile forms the central mass of the ventral half of the cord. It contains longitudinally and transversely running nerve-fibres, among which may be distinguished fibres which (1) extend from one segment to the next; (2) cross the nerve-cord; (3) enter into the peripheral nerves; (4) connect with nerve-cells in the cord; (5) join the giant nerve-fibre. The supporting tissue is composed of very slender fibrils and small cells which lie among the nerve-cells and about the giant nerve-fibre.

### (b) *Arrangement of the giant axon*

The giant nerve-fibre forms the most conspicuous and the predominating feature of the central nervous system of this species. It arises in the supra-oesophageal ganglia, extends down the oesophageal connectives into the

PLATE 1. *Myxicola infundibulum*

All figs. on this plate are from sections of material cleared in cedarwood oil and embedded in paraffin wax, unless otherwise stated.

Fig. 1. T.S. of the nerve-cord showing the giant axon and the origin of the peripheral nerves from the cord. Helly's, Holmes's silver, Heidenhain-azan.

Fig. 2. Horizontal longitudinal section through the supra-oesophageal ganglia showing the decussation of the two giant axons in this region. Helly's, Holmes's silver, Heidenhain-azan.

Fig. 3. Horizontal longitudinal section of the nerve-cord. Continuity of axoplasm of the giant fibre and a peripheral branch is shown clearly. Picro-formol, Péterfi's celloidin-paraffin, Holmes's silver.

Fig. 4. Sagittal section showing two successive branches of the giant axon. The fibre from the right-hand branch extends longitudinally through the nerve-cord, and joins the left-hand branch in the next section. Picro-formol, Péterfi's celloidin-paraffin, Holmes's silver.

Fig. 5. T.S. of the giant fibre showing striations in the axoplasm. Flemming's (without acetic), iron haematoxylin and orange G.

Fig. 6. Composite picture built up by superposition of photomicrographs of three serial and consecutive sections. The giant fibre branch on the left subdivides into three fibres, and also sends a fibre diagonally across the nerve-cord towards the opposite side. Helly's, Holmes's silver, Heidenhain-azan.

nerve-cord, and runs through the entire length of the latter structure (Text-fig. 1A).

Within the supra-oesophageal ganglia the giant fibres lie in the ventro-posterior border of the inferior lobes (Pl. 1, fig. 2, and Pl. 3, fig. 14). They are very slender, about $15\mu$ in diameter, and extend transversely through these ganglia across the mid-line. Near the median plane they are connected with two relatively large cells which occur ventrally and anteriorly to them, and which also have their axes of greatest length arranged transversely (Pl. 3, fig. 15). On descending into the oesophageal connectives each fibre gives off a number of branches which proceed dorsally and anteriorly within the neuropile of the supra-oesophageal ganglia and oesophageal connectives. The fibres lie on the median face of the connectives and, in the sub-oesophageal ganglion, the two fibres fuse together in the mid-line (Text-figs. 3A and 3B).

A short distance in front of the point where the oesophageal connectives arise from the anterior end of the nerve-cord they are connected together by a narrow transverse bridge. This commissure contains at least one fibre which connects together the giant fibres of each side. It is difficult to resolve the true picture here because of the multitude of fibres present, but in horizontal longitudinal sections it can be seen definitely that a slender branch from the giant fibre in each oesophageal connective curves ventrally and posteriorly, passes into this commissure, and meets a corresponding fibre from the other side in this latter structure.

In thoracic segments II to IV the giant fibre usually branches where the nerve-cord divides into two and a fibre runs through each half of the nerve-cord (Text-figs. 1A and 4A). The two fibres so formed join together where the two cords fuse or, to use the conventional nomenclature, they are connected together by commissures. But when one of the strands of the nerve-cord is smaller than the other, as frequently happens, the giant fibre may extend through one strand of the cord only and thus remain single even when the cord is divided. The pattern of the giant fibres in the thorax thus shows a considerable amount of individual variation and it is impossible to present a rigid description that is applicable to every case. At those places where the giant fibres bifurcate and fuse again it is certain that there is continuity of axoplasm, and that the several portions are united to one another without the interposition of intervening septa or separating membranes (Text-fig. 4A). This is clearly seen in all preparations, fixed and stained by different methods, and cut in three planes at right angles to one another. Posterior to segment IV the giant fibre remains single and median in position throughout its length (Text-fig. 4B).

The following figures, obtained from a mature specimen, are representative of the relative diameters of the giant fibre in different regions of the body. The giant fibres are small in size in the dorsal half of the oesophageal connectives (about $60\mu$), increase in diameter as they pass ventrally (about $70\mu$), and, just before fusing in the sub-oesophageal ganglion, each fibre attains a diameter of about $100\mu$. Immediately after the fibres fuse the diameter is

200$\mu$. The fibre continues to expand in the thorax, having a width of 300$\mu$ where single and a combined diameter of about the same where bipartite. It reaches its greatest width in the posterior thorax (500$\mu$), then gradually decreases to 100$\mu$ in the middle of the abdomen and very gradually tapers off posteriorly.

The figures given above are not constant, for there is a considerable amount of individual variation (in one case a diameter of 1·7 mm. was measured), and the measurements obtained also depend on whether the animal was killed in a contracted or extended state. In order to determine the variation in size of the giant axon in animals of different lengths and the alteration in diameter that occurs during contraction and extension, a number of animals of different sizes were killed in extended and contracted conditions. Sample sections were cut at regular intervals along the length of these specimens and the maximal horizontal diameters of nerve-cord and giant axon were measured and plotted against length. The results are shown in Text-fig. 6. To afford an indication of the size of the animal its extended and contracted length were determined before death and are marked on the left-hand side of each figure. Text-fig. 6A represents the nerve-cord and giant axon of the largest animal, abnormally extended under anaesthesia. The giant axon has a maximal diameter of 560$\mu$ in the thorax and decreases to 140$\mu$ in the mid-abdomen. B and C were obtained from smaller specimens of about equal size. In these animals the giant axons have maximal diameters of 1·3 and 0·7 mm. in the anterior nerve-cord. D and E represent small animals with correspondingly small giant axons. The extreme variation shown by the results was quite unexpected and theoretically can be ascribed to two factors: (1) variation in the size and shape of the giant fibre in different animals, even of approximately the same size; (2) variation in the extent of contraction of different body regions during fixation. The following facts are apparent, however. The giant axon has a greater diameter in larger than in smaller animals. Its diameter is maximal in the posterior thorax or anterior abdomen and decreases posteriorly. The diameter of the giant axon increases greatly during the contraction of the animal. It may be concluded that in any study of the conduction properties of this axon, each animal will have to be treated as an independent unit, and the shape and size of its giant axon determined.

A further study was made of the variation in size of the giant fibre relative to the volume of the nerve-cord and the total body volume. This is shown in Text-fig. 7. These figures were obtained by making a series of camera lucida drawings of the parts concerned at several levels, cutting out and weighing the pieces of paper, and multiplying the mean of the weights so obtained for each section of the animal by the length of that section (the method, of course, is based on the assumption that all parts of the animal have the same density and makes no allowance for body fluids). The resultant figures are summarized in Table 1. The giant fibre occupies only a relatively small proportion of the anterior prostomial and oesophageal mass of nervous tissue, viz. 1·1 per cent., while, posterior to this region, it forms 32 per cent. of the
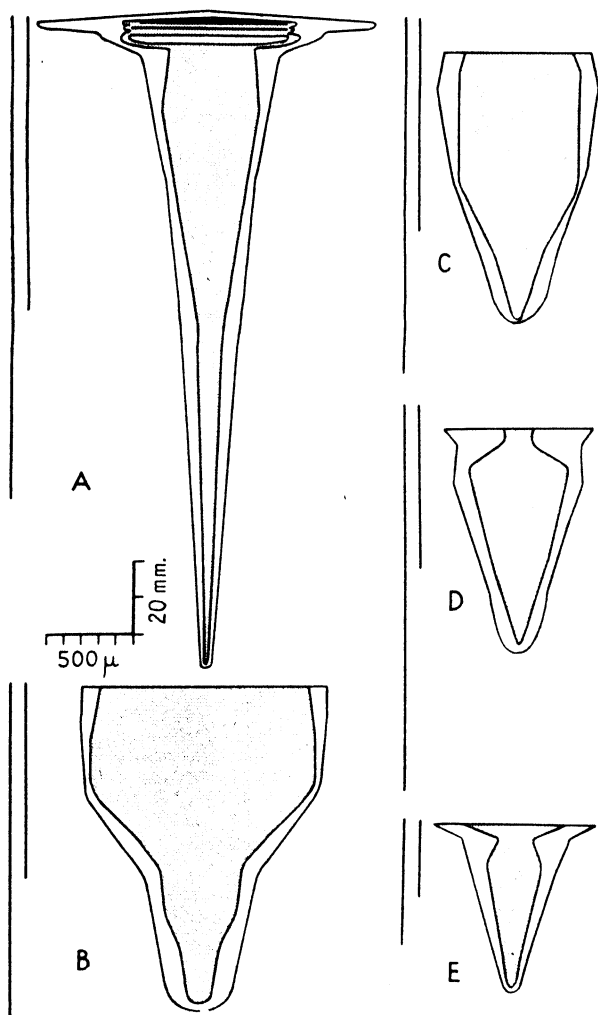
TEXT-FIG. 6. Reconstructions to scale of the nerve-cord and giant axon of five specimens of *M. infundibulum* fixed in different states of contraction and expansion: A, extended by alcohol anaesthesia; B–E, partially contracted by the fixative. The figures were drawn from maximal horizontal diameters of the structures concerned. The external line in each figure represents the boundary of the nerve-cord, the internal shaded area the giant axon. The anterior thoracic and oesophageal regions have been determined in specimen A only; the remaining figures begin at about the level of the fourth setiger. The two lines to the left of each figure indicate normal lengths of the entire worm when extended and contracted prior to death.

TABLE I. *Relative volumes of body, nerve-cord, and giant fibre*

1. Region of prostomium and setigers I and II (containing supra-oesophageal ganglia, oesophageal connectives, and sub-oesophageal ganglion).

|  |  |
|---|---|
| Body volume . . . | 3,847·6 |
| Nerve-centre volume . . . | 130·8 |
| Giant fibre volume . . . | 1·5 units |

2. Thoracic and abdominal regions (combined results).

|  |  |
|---|---|
| Body volume . . . | 65,967·6 |
| Nerve-cord volume . . . | 665·1 |
| Giant fibre volume . . . | 231·3 units |

3. Ratios.

A. Prostomium and setigers I and II.

$$\frac{\textit{Anterior nerve centres}}{\text{Body volume (less crown)}} = 0.036 = 3.6 \text{ per cent.}$$

$$\frac{\textit{Giant fibre}}{\text{Anterior nerve-centres}} = 0.011 = 1.1 \text{ per cent.}$$

$$\frac{\textit{Giant fibre}}{\text{Body volume (less crown)}} = 0.0005 = 0.05 \text{ per cent.}$$

B. Thorax and abdomen, posterior to setiger II.

$$\frac{\textit{Nerve-cord}}{\text{Body volume}} = 0.0109 = 1.1 \text{ per cent.}$$

$$\frac{\textit{Giant fibre}}{\text{Nerve-cord}} = 0.319 = 31.9 \text{ per cent.}$$

$$\frac{\textit{Giant fibre}}{\text{Body volume}} = 0.003 = 0.3 \text{ per cent.}$$

C. Entire animal (less branchial crown).

$$\frac{\textit{C.N.S.}}{\text{Body volume}} = 0.01 = 1 \text{ per cent.}$$

$$\frac{\textit{Giant fibre}}{\text{C.N.S.}} = 0.266 = 26.6 \text{ per cent.}$$

$$\frac{\textit{Giant fibre}}{\text{Body volume}} = 0.003 = 0.3 \text{ per cent.}$$

volume of the nerve-cord. This single fibre constitutes, therefore, slightly more than 25 per cent. of the entire central nervous system of the animal (exclusive of the branchial crown). The curves shown in Text-fig. 7 bring out two further points: behind the sub-oesophageal ganglion the cord has a nearly constant volume, relative to that of the body; the giant fibre, on the other hand, relative to both cord and body volume, decreases considerably caudally.

## (c) Nerve-cells

Careful examination of serial sections has shown that the giant fibre is connected with nerve-cells throughout its length. The nerve-cells of the cord vary in diameter from about $10\mu$ to $50\mu$ and are elongate or pyriform, the majority being somewhat ellipsoid in shape. They appear to be uniformly unipolar since in no case was more than one cell process seen. In appearance all the cells of the cord are of the same type. The cytoplasm appears finely granular;

there are no distinct neurofibrillae. The nuclei are oval or reniform, about $9\mu$ in diameter, contain a single, basiphilic nucleolus, about $3\mu$ in diameter, and sparse granules or flakes of chromatin. The cell process arises from a
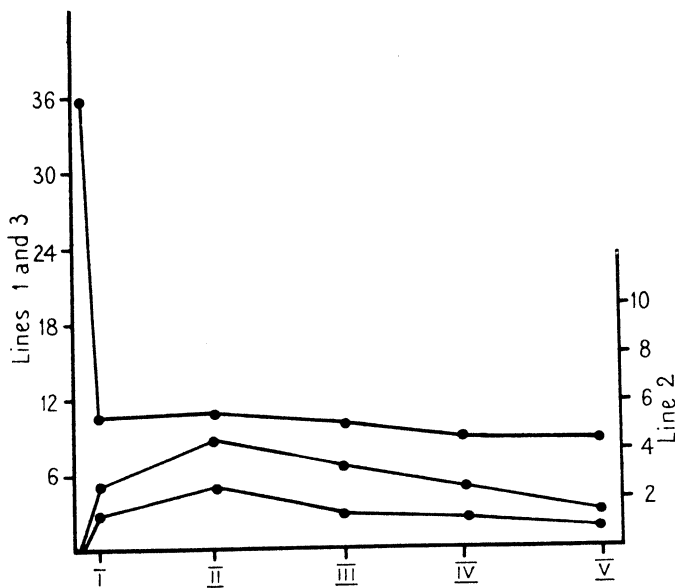


TEXT-FIG. 7. Curves showing the relative volumes of giant axon, nerve-cord, and body of *M. infundibulum*.

Ordinates:

Line 1, $\dfrac{CNS}{\text{Body volume}} \times 100$

Line 2, $\dfrac{Giant\ fibre}{CNS} \times 10$

Line 3, $\dfrac{Giant\ fibre}{\text{Body volume}} \times 1{,}000$

Abscissae:

I. Prostomium and setigers I and II; II. thorax; III. anterior abdomen; IV. middle abdomen; V. posterior abdomen.

swollen base and contains fine longitudinal fibrillae. The most characteristic feature of the nerve-cells is the presence of numerous vacuoles which are of constant occurrence. Typically each cell contains one or several large vacuoles and a number of smaller ones arranged peripherally about the cell (Pl. 2, fig. 10).

Nissl material may be seen in cells fixed in Carnoy fluid or picro-formol and stained by various standard methods, including toluidine blue and Einarson's gallocyanin. Although groups of nerve-cells in the supra-oeso-phageal ganglia contain deeply staining masses of tigroid, all the nerve-cells

of the cord contain Nissl material in the form of diffuse and extremely fine granules dispersed throughout the cell. These granules occur in both the perinuclear zone and in the peripheral cytoplasm investing the vacuoles; they are absent from the cell process.

Although all the nerve-cells of the cord have a similar appearance, a non-critical examination of the sections gives the impression that some cells are distinctly larger than the others and have stouter processes and larger nuclei. The possibility therefore arises that different types of nerve-cells may be present in the cord and that they can be distinguished on the basis of size differences rather than of differences in form or content and that among these types may reside the nerve-cells of the giant fibre.

This hypothesis was worth investigating since it has been suggested frequently, and proved in some cases, that the giant fibres of Polychaetes arise from particularly large nerve-cells. Accordingly, cell and nuclear diameters of all nerve-cells were measured in a set of serial transverse sections extending through about one segment in the anterior abdominal nerve-cord. To avoid measuring the same cell twice only those cells were measured that contained nucleoli. In all, about 200 cells were measured and the results are shown in Text-figs. 8 and 9. Maximal and minimal diameters of cells and nucleoli were determined and the means of these two diameters were plotted against frequency. The resulting histograms indicate that the size variation for the cells is a unimodal one. The cells vary in size from $10\mu$ to $50\mu$; the mean is $25\mu$ ($\sigma$ 9; se 9·63). Nuclei range in size from $4\mu$ to $16\mu$; the mean is $9\mu$ ($\sigma$ 2·5; se 0·17).

*Nerve-cells of the giant fibre.* The nerve-cells of the giant fibre are of two types, namely, (1) two large cells in the supra-oesophageal ganglia, each of which joins one of the two fibres in this region, and (2) numerous cells which

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PLATE 2. *Myxicola infundibulum*

Fig. 7. T.S. of the ventral half of the nerve-cord. Baker's Ca-formol, frozen section, Sudan black.

Fig. 8. A low-power view of the ventral half of the body by the same technique.

Fig. 9. T.S. of part of the nerve-cord and giant fibre. The photograph is orientated with upper margin corresponding to the dorsal side. Picro-formol, cedarwood oil, paraffin, Holmes's silver, safranin.

Fig. 10. T.S. of part of the nerve-cord to show a giant fibre cell. Orientation, right-hand side of photograph corresponding to dorsal side. Picro-formol, cedarwood oil, paraffin, Holmes's silver.

Fig. 11. A composite picture built up from three photographs of three successive serial sections. From the ventro-lateral region of the nerve-cord. The result shows a nerve-cell joining a peripheral branch of the giant fibre in a T-shaped junction. The axoplasm is very diffusely and weakly impregnated at the point of fusion between the cell process and the peripheral branch. Picro-formol, cedarwood oil, paraffin, Holmes's silver.

Fig. 12. T.S. of ventro-lateral region of the nerve-cord showing a peripheral branch passing from the giant fibre into the body-wall. This photograph should be compared with fig. 3 in order to understand the difficulty involved in determining whether the peripheral branches are actually continuous with the giant fibre or make synapse with it. The difference in degree of silver impregnation between the giant axon and its branch in this section affords no indication of fusion. Picro-formol, cedarwood oil, paraffin, Holmes's silver.

are distributed along the length of the nerve-cord. The giant fibre cells in the nerve-cord will be considered first.

The above study reveals that the giant fibre cells in the cord have no special peculiarities which distinguish them from the other cells of the cord: they are not giant cells and they have the same nuclear and cytoplasmic features as the other nerve-cells. They are unipolar, often elongate, and highly vacuo-
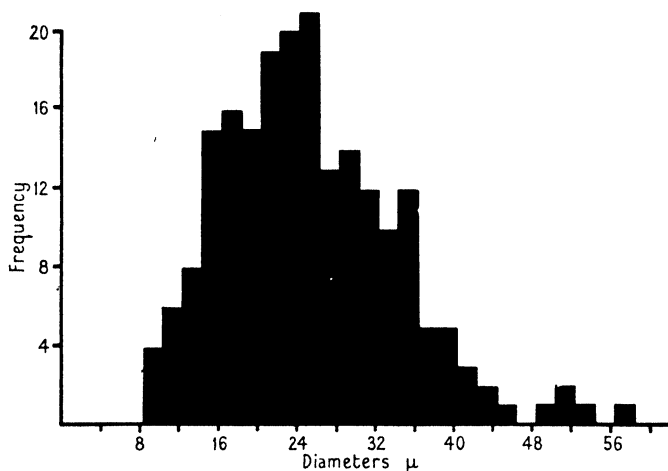


TEXT-FIG. 8. Histogram showing variations in the size of nerve-cells in the nerve-cord of *M. infundibulum*. Population = 209. Ordinates, frequency; abscissae, diameters in $\mu$ (means of two maximal measurements).

lated, frequently with one or two large vacuoles at each end of the cell. Occasionally they lie ventrally in the nerve-cord, but they are usually seen in a ventro-lateral position. No weight is attached to this observation, however, for it has not been possible to determine all the cellular connexions of the giant fibre and in any series of sections many were certainly overlooked.

The nerve-cells are connected with the giant fibre in two ways: either directly, the cell process joining the giant fibre itself; or indirectly, the cell joining the lateral branches which pass to the periphery of the body. In the first case the cell sends a longer or shorter process through the neuropile of the nerve-cord and this process enters the giant fibre somewhere on its ventral surface after penetrating its fibrous sheath. Frequently, the point of fusion is at the ventro-lateral margin of the giant fibre (Pl. 2, fig. 10). In the second case, a distinct cell process may pass through the nerve-cord to join the lateral branch (Pl. 2, fig. 11); or the cell may lie immediately beside the branch and its cytoplasm and the axoplasm of the lateral branch may fuse with one another over a broad zone of junction.

Because of difficulties in staining all the cell processes and following their course through several serial sections, it has not been possible to enumerate the number of nerve-cells connected with a given length of the giant fibre,

but it is certain that they must be fairly numerous.  One to three cells have been seen to fuse with each lateral branch.  If there be eight lateral branches per segment, each connected with one nerve-cell; and two additional cells join the giant fibre independently of the lateral branches in each segment; and the animal possesses 130 segments: then the giant fibre throughout its course in the nerve-cord would be connected, either directly or indirectly,
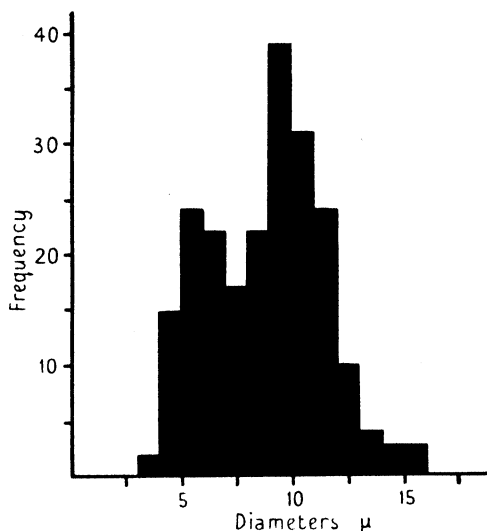


TEXT-FIG. 9. Histogram of size variation of nerve-cell nuclei in the nerve-cord of *M. infundi-bulum*. Population = 214. Ordinates, frequency; abscissae, diameters in $\mu$ (means of two maximal measurements).

but in any case without the interposition of a separating membrane, with 1,300 nerve-cells.  It is certain that this figure is a conservative estimate. There are about 300 nerve-cells per segment in the anterior abdomen and thorax of a mature worm and probably about 30,000 in the entire nerve-cord. At least 5 per cent. of the cells present in the nerve-cord, therefore, represent the nerve-cells of the giant fibre and its branches.  It is not possible to be more accurate than this, but it is probable that the giant fibre cells are more numerous than the figures given.

The nerve-cells lie among the supporting fibres and cells of the cord and, unlike the giant fibre, lack distinctive investing sheaths.  Occasional small cells, with scanty cytoplasm, lie on the nerve-cells like small, ragged tags, but they do not form complete capsules.  And although the giant fibre sheath is recurved over the surface of incoming nerve processes and the peripheral branches, it covers them for a short distance only before merging into the surrounding supporting tissue.

The giant fibre cells of the supra-oesophageal ganglia are very characteristic structures, when seen in coronal sections (Pl. 3, fig. 15).  There are usually

two of them, symmetrically arranged beside one another in a transverse plane, at the extreme ventro-posterior end of the inferior ganglia. Typically these cells are very long and narrow. Each cell extends from the median sagittal plane through the commissural bridge uniting the two inferior ganglia, into the neuropile of the latter. The cell dimensions are about $120\mu \times 30\mu$. The nucleus lies centrally in the cell and is oval or nearly round, $25\mu \times 20\mu$ in diameter. There is a single eccentric nuceolus, $5\mu$ in diameter, and a conspicuous round vacuole, about $4\mu$ in diameter, in the nucleus. The nucleolus stains densely and is basiphilic. The vacuole fails to stain and is surrounded by a rim of dark granules. The cytoplasm consists of a slightly more densely staining central portion closely surrounding the nucleus, and having the appearance of a fine reticulum, the main cytoplasm in the centre of the cell which contains numerous, fine fibrils arranged parallel to the long axis of the cell, and a peripheral, vacuolated zone containing numerous vacuoles of various sizes resembling those of the nerve-cells of the cord. The fibrillae of the cell cytoplasm extend out into the cell process. The cell is apparently unipolar and sends a conspicuously large process from its median end obliquely dorsally and posteriorly to the giant fibre. The process at its origin from the cell has a diameter equal to that of the cell, and twice that of the giant fibre to which it is proceeding; it gradually diminishes from about $30\mu$ to about $15\mu$ before joining the giant axon.

It has been stated above that the giant fibres in the supra-oesophageal ganglia approach each other medially. It is difficult to determine whether they fuse or decussate with one another in transverse sections. They appear to decussate in horizontal longitudinal sections. Careful study and reconstructions of the giant fibre and its cells in sagittal sections show that it is the latter condition which obtains, an arrangement that was quite unexpected and that is completely at variance with the extraordinary amount of interneuronal fusion occurring throughout the remainder of the length of the giant fibre. The two fibres lie in very close apposition in the mid-line, and are separated from one another by their surface membranes only. They clearly twist about each other through an angle of $180°$; the fibres remain discrete; they soon separate again and each fibre can be seen to arise as the cell process of a contralateral giant fibre cell (Text-fig. 10; Pl. 1, fig. 2, and Pl. 3, fig. 15). The giant nerve-fibre, therefore, arises from a pair of large nerve-cells in the supra-oesophageal ganglia. The single processes of these two cells decussate with each other in the mid-sagittal plane, and each process passes down the oesophageal connective contralateral to the cell of origin. It is only in the sub-oesophageal ganglion that the two giant fibres fuse together for the first time.

## (d) Axoplasm

When examined in the living animal the giant fibre appears to be completely homogeneous without any indication of microscopic fibrillae or striations. In this regard it is probably significant that Young (1936) failed to find

any clearly marked neurofibrillae in the giant axons of Cephalopods, although definite fibrillae appeared after injury to the nerve-fibre. In preparations fixed and stained by various methods, including both protein precipitants such as picric acid, and non-protein precipitants such as formaldehyde and osmium tetroxide, delicate longitudinal striations can be seen in the axoplasm. Such striations are very fine and are near the limit of optical resolution in the visible spectrum; they are very short, moreover, and are not organized into definite threads that can be followed for any distance. Fine, circularly arranged fibrils
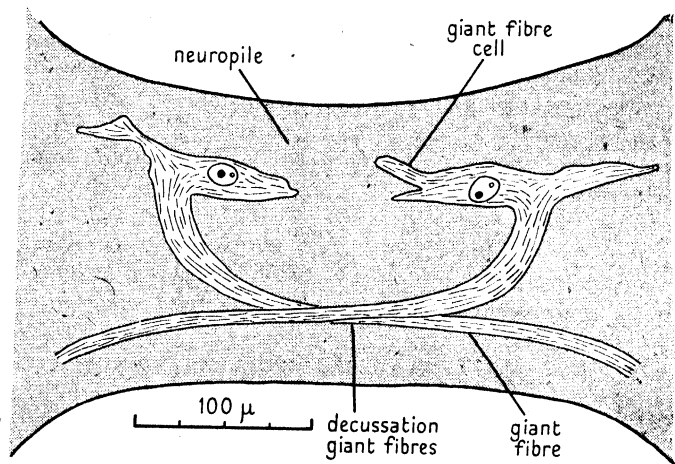


TEXT-FIG. 10. Reconstruction of the giant fibres and their cells in the supra-oesophageal ganglia, based on camera lucida drawings. The cells and the level of decussation are shown in the same plane; actually, the nerve-cells of the giant fibres in this region lie ventrally to the latter.

can be seen also in transverse sections, and these tend to form a definite pattern in that the mat of fine fibrillae lies parallel to the external surface of the axon (Pl. 1, fig. 5). These patterns of fibrillation are particularly well marked near the cut end of an axon and seem to conform to the direction of axoplasmic flow. A somewhat similar configuration was found by Young (1936) in Cephalopod giant axons where fine longitudinal striations could be seen in sections after treatment with various histological reagents. In the case of Polychaetes, Ashworth (1909) stated that the intracellular neurofibrillae of the giant fibre-cells of Halla passed out into the axons, and Cunningham (1888) found that the giant fibres of Sabella were refractory to staining except for fine lines resembling fibres in the axoplasm. Striations of this sort possibly represent a preformed micellar organization of the axoplasm for which evidence has been advanced by Bear, Schmitt, and Young (1937) as the result of birefringence studies.

A very interesting feature of the giant fibre of Myxicola in life is that a

length of this structure may be squeezed out readily from the nerve-cord of the thoracic and anterior abdominal regions. This is best accomplished by cutting open the dorsal surface of the animal, removing the gut, and dissecting free a length of ventral body-wall containing the nerve-cord. When a glass rod is pressed along such a preparation, removed from sea-water, and placed on a glass plate, axoplasm is extruded from the giant axon of several segments. Probably the entire axon is pressed out by this means since its substance is rather viscous and retains clearly the shape that it possesses within the body. In fact strands 3–4 cm. long were obtained on a few occasions by this means and they retained their threadlike form for several minutes before collapsing. It is clear that the axoplasm of this species is much more viscous than that of squid giant fibres (Young, 1934, 1936) and shows little tendency to flow. Whether the viscosity which is revealed very crudely by this means is sufficiently low to permit the great changes in shape of the nerve-fibre that occur in the living animal, or whether there is an alteration in the viscosity of the axoplasm during various phases of activity, must await further investigation.

## (e) Sheath

The giant fibre is surrounded by a distinct sheath which envelops it on all sides. This sheath is about $5\mu$ to $15\mu$ thick and consists largely of a dense meshwork of fine fibrils arranged in a predominantly circular direction about the fibre. Within it are spaced small flattened nuclei at considerable intervals. The fibrils of the sheath are very slender and peripherally are continuous with the fibrous network of the rest of the nerve-cord. They do not appear to be collagenous, they stain poorly with all stains, including connective tissue stains, and they are only slightly darkened by the silver techniques used in this study. The cytoplasm of the supporting cells is probably scanty, but it is impossible to obtain any clear idea of form since the cell-body fails to stain. It is only occasionally that a small tag of protoplasm can be seen about a nucleus. The nuclei are flattened, about $8\mu \times 4\mu \times 2\mu$, with the long axis tangential to the surface of the fibre. Finally, the sheath clings very closely to the giant fibre and there is no indication of any intervening space or cellular layer.

This sheath does not darken when treated with osmium tetroxide, a result which would indicate that myelin, if present, is very scanty in amount. Neither is there any indication of lipines in the sheath when sections are carried through Baker's acid-haematein test. This test does reveal, however, a marked concentration of lipines on or in the peripheral nerves, and in the epidermal cells. The positive blue colour is very striking, and extends into the neuropile of the nerve-cord along the nerve-fibres. Sections stained with Sudan, moreover, give very interesting results (Pl. 2, figs. 7 and 8). In the case of Sudan black, there is a very dense concentration of dye in the subperitoneal tissue and extending into the longitudinal muscles, and lesser concentrations in the epidermal cells, epithelial cells of the gut, in the peripheral nerves, and in

the nerve-cord. Some of the epidermal cells are very heavily coloured and contain dense masses of dye in their peripheral portions. The dye is rather diffusely distributed in the nerve-cord; it is evenly distributed within the cytoplasm of the nerve-cells and there is a slight but clearly distinguishable concentration in the sheath of the giant fibre, in a band about $10\mu$ to $15\mu$ in thickness. The peripheral nerves are deeply coloured, and this coloration clearly extends into the nerve-cord. The peripheral nerve-fibres are very fine and it is not certain where this fatty material is localized; but since the axoplasm of the giant fibre and of its peripheral branches remains negative it is reasonable to assume that the lipoids are concentrated about the peripheral fibres. Somewhat similar results are given by the use of Sudan IV, but the coloration of the nervous system in this case is very light, and gives no indication of a fatty sheath about the giant fibre. Mounted sections were immersed for 1 hour in alcohol-ether and then treated with Sudan black. It was found after this treatment that the dense, subperitoneal deposits of lipoids were largely removed. This tissue probably consists of a layer of fatty cells loaded with triglycerides. The epidermal cells were apparently unaffected by this treatment, while the coloration of the nervous system was considerably reduced and it was no longer possible to distinguish any accumulation of fatty material about the giant fibre. It may be concluded, therefore, that the peripheral nerves are probably thinly myelinated and are invested by deposits of lipoids, in the broad sense, including lipines, and that there is a thin lipoidal sheath about the giant axon (cf. Bear, Schmitt, and Young, 1937).

### (f) Peripheral branches

The giant fibre gives off conspicuous peripheral branches along its entire length in the nerve-cord and these branches are distributed to the longitudinal musculature. The branches have typically the following pattern. About four lateral fibres arise from each side of the giant fibre in each segment (Text-fig. 1B). From this position of origin, at a point on the ventro-lateral surface of the giant fibre, they extend obliquely ventrally across the cord to enter the peripheral nerves (Text-fig. 11; Pl. 2, fig. 12). Since there are about twelve peripheral nerves in each segment and only about eight giant fibre branches, some of the peripheral nerves lack these structures. They occur in all of the larger nerves, however. The giant fibre branches divide in a complex manner within the nerve-cord before emerging peripherally. These subdivisions are distributed so as to (1) form additional junctions with the giant fibre, either laterally, medially, or dorsally, (2) unite the giant fibre branches of each side of the nerve-cord, (3) join successive giant fibre branches within the cord, or (4) connect with nerve-cells. Not all of these arrangements have been seen in any one segment or part of a segment, but they have been found sufficiently often to show that the above pattern is typical.

*Origin of the peripheral branches.* The manner of origin of these efferent branches from the giant fibre shows a great deal of variation and complexity.

These branches not only enter the peripheral nerves but also form an anastomosing web within the nerve-cord, the different branches fusing with one another. Because of their extreme variation in size, the complex courses that they follow and the fact that only the best preparations show clearly their origin from the giant axon, this study has been very difficult.

Usually a peripheral branch of the giant fibre arises by three or more roots from the main fibre, each root rather small, $2\mu$ to $10\mu$, but more rarely much greater than this, exceptionally as much as $60\mu$ in diameter (Pl. 2, fig. 12).
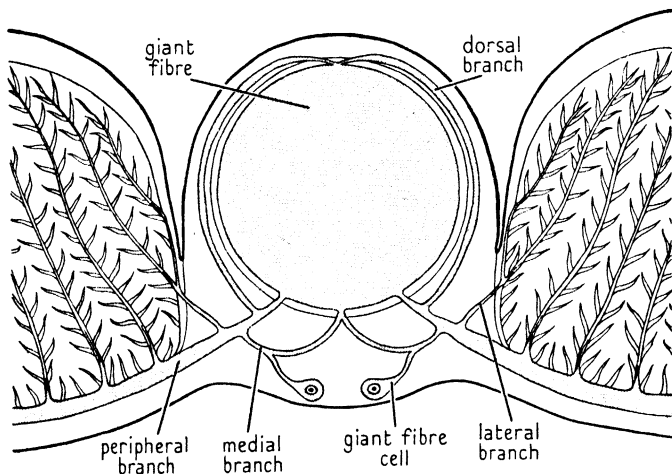


TEXT-FIG. 11. Diagram of the nerve-cord of *M. infundibulum,* as seen in transverse section. Note dorsal branch of the giant fibre; lateral branch of giant fibre to longitudinal muscle-fibres lying beside the nerve-cord; peripheral branch of the giant fibre. The pennate structures on each side of the nerve-cord represent longitudinal muscles.

The several processes form a root-like aggregation on the ventro-lateral face of the giant axon, fusing with the latter close to one another. Since they tend to be arranged in line, longitudinally, the determination of their arrangement from transverse serial sections entails tedious reconstructions that introduce an element of doubt owing to their complexity. This picture is fully confirmed by horizontal longitudinal sections that reveal this group of branches in a single section.

A superficial examination gives the impression that the peripheral branches are separated by membranes from the giant fibre since there is frequently some suggestion of an intervening structure at the place of junction. This point was carefully examined in serial sections sagittally, frontally, and transversely in material embedded in celloidin and Péterfi's celloidin-paraffin as well as in paraffin sections. Fortunately, such points of junction are frequently very large in some of the thoracic segments (up to $60\mu$ in cross-section) and can be easily studied. In many cases it was clearly apparent that there

is definitely a fusion of substances and the axoplasm of the giant fibre is continuous with that of the peripheral branch over a wide area (Pl. 1, fig. 3).

*Giant fibre branches within the nerve-cord.* A peripheral branch of the giant axon crosses the neuropile and enters a peripheral nerve. In its passage ventrally through the neuropile it gives off a number of twigs. It is known that some of these twigs have the following arrangement: (1) A process from the peripheral branch extends transversely through the neuropile and forms an additional junction with the giant axon somewhere in the median plane, either singly, or in conjunction with a similar process from the opposite side of the nerve-cord (Pl. 1, fig. 6). (2) A process extends longitudinally through the neuropile to fuse with the next successive peripheral branch of the same side of the nerve-cord (Pl. 1, fig. 4). (3) A process extends dorsally from the main peripheral branch up to even beyond the median dorsal face of the nerve-cord (Pl. 2, fig. 9). Such dorsal processes may expand somewhat as they pass dorsally beside the giant axon and may be quite large, $8\mu$ to $10\mu$ in diameter. An occasional small nerve-cell occurs here and there dorsal to the giant fibre, but it has not been possible to find any connexion between the dorsal processes and such nerve-cells. Moreover, this arrangement appears to be symmetrical in that a dorsal process arises at the same transverse level on each side of the nerve-cord. The processes diminish in size dorsally, approach each other, and terminate on the surface of the giant fibre. The conclusion has been reached that they fuse with one another and with the giant fibre dorsally. (4) Processes of the peripheral branch join nerve-cells in the ventral region of the nerve-cord.

*Peripheral distribution of the giant fibre branches.* The peripheral branches show considerable size variations in the nerve-cord, but they become more uniform after entering the nerves proceeding to the body-wall (Text-fig. 11; Pl. 2, fig. 12, and Pl. 3, fig. 18). They are about $40\mu \times 85\mu$ in the thoracic and anterior abdominal nerves of a mature animal and become smaller more posteriorly. Frequently, before emerging from the nerve-cord they give rise to a small lateral twig, which perforates the lateral wall of the nerve-cord above the peripheral nerves proper, and which proceeds directly to the group of longitudinal muscle-fibres which lies immediately beside the nerve-cord, at some distance from the body surface. The peripheral nerve which the lateral branch enters may contain a larger or smaller bundle of small nerve-fibres as well, or may consist of the giant fibre branch only. This branch then proceeds dorsally, between the circular and longitudinal muscle layers, immediately beneath the connective tissue lamella investing the latter, diminishes gradually in diameter, and disappears near the medio-dorsal surface of the animal.

The peripheral nerves are invested by a thin sheath of connective tissue. The branches of the giant fibre may be the sole component of the peripheral nerves, or smaller motor and sensory fibres may accompany them. Bundles of smaller fibres frequently traverse the circular muscle layer and penetrate the epidermis where they lie among the bases of the epidermal cells, but the

branches of the giant fibre maintain a constant position beneath the layer of longitudinal muscles.

The longitudinal musculature of *M. infundibulum* has been described briefly by Claparède (1873) and Johansson (1927). It is very strongly developed and extends the entire length of the animal. The muscle-fibres themselves consist of longitudinal ribbons flattened in transverse section and are arranged along a central axis in a manner that gives the muscle layer a typical pennate appearance when seen in transverse section. The central axes or stalks radiate inwards from the periphery and contain nerve-fibres and a small number of connective tissue fibres which are continuous with the thin sheet of connective tissue underlying the whole longitudinal muscle layer (Text-fig. 11; Pl. 1, fig. 1). The fibres are constructed on the 'nematoid' pattern (cf. Stephenson, 1930), and consist of two closely apposed contractile plates, with a thin intervening layer of sarcoplasm. The peripheral branches of the giant fibre lie directly beneath the layer of longitudinal muscles and over short stretches the intervening connective tissue lamella disappears and the bases of the muscle-fibres rest directly on the nerve-fibres (Text-fig. 11; Pl. 3, fig. 16). However, only a small proportion of the muscle-fibres lies peripherally on the connective tissue lamella underlying the layer of longitudinal musculature; the majority are attached like barbs to the radiating stalks and slender twigs of the peripheral branches of the giant fibre enter these stalks. It has not been possible to follow these secondary branches for any distance into the stalks because they become very slender and disappear from sight. They are about $4\mu$ in diameter where they enter the stalks and soon dwindle to very slender strands. Nervous structures among the muscle-

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PLATE 3

All preparations cleared in cedarwood oil and embedded in paraffin wax.

Figs. 13–16 represent *Myxicola infundibulum*, Figs. 17–18 *M. aesthetica*.

Fig. 13. T.S. through the trunk in the anterior abdominal region. The position of the giant fibre in the dorsal part of the nerve-cord and the large volume of the longitudinal muscles are well shown. Formol, cedarwood oil, paraffin, Holmes's silver, light green.

Fig. 14. Horizontal longitudinal section of supra-oesophageal ganglia showing two giant fibres. Helly's, protargol, Heidenhain-azan.

Fig. 15. Another section from the same animal, more ventral to that shown in Fig. 14.

Fig. 16. T.S. through body wall showing branch of giant fibre lying immediately beneath the layer of longitudinal muscles. Helly's, protargol, Heidenhain-azan.

Fig. 17. Horizontal longitudinal section through sub-oesophageal ganglion and anterior nerve-cord, showing giant fibre bifurcating and entering the oesophageal connectives. Picro-formol, Holmes's silver.

Fig. 18. Another section through the nerve-cord further caudad to Fig. 17 in the same animal.

*Legend*: *c*, nerve-cord; *d.b*, dorsal branch of the giant fibre; *d.g.f*, decussation of two giant fibres in the supra-oesophageal ganglia; *e*, epidermis; *g*, gut; *g.f*, giant fibre; *g.f.c*, nerve-cell of the giant fibre; *g.f.s*, sheath of the giant fibre; *i.l*, inferior lobes of the supra-oesophageal ganglia; *l.b*, longitudinal branch of the giant fibre; *l.m*, longitudinal muscle; *m.b*, median branch of the giant fibre; *n*, neuropil; *n.c*, nerve-cell(s); *n.c.f*, process of giant fibre nerve-cell; *n.s*, sheath of the nerve-cord; *p.b*, peripheral branch of the giant fibre; *p.b.f*, twig of peripheral branch extending into longitudinal musculature; *p.n*, peripheral nerve; *p.n.f*, peripheral nerve-fibres ending in neuropile; *v*, vacuole in nerve-cell; *v.m*, ventral mesentery.

fibres that could be interpreted as terminal expansions, plates, or even branching terminations of nerve-fibres have never been observed. It is possible that the connexion between the nerve- and muscle-fibres is one of simple contact, since the membranes of the two structures are in intimate contact at those points where the nerve-fibres pass beneath the muscle-fibres (Pl. 3, fig. 16).

The innervation of the longitudinal musculature in the anterior thoracic segments is essentially the same as that described above, but a special case arises when one of the halves of the nerve-cord lacks a giant fibre. There is no evidence that the peripheral branches of the giant fibres cross the mid-dorsal line, and it is probable that the longitudinal musculature of each half of the body receives its nerve-supply exclusively from the nerve-cord of that side. However, in cases where the giant fibre is not present in a lateral strand of nerve-cord in the thorax, it has been observed that a peripheral branch of the giant fibre extends a considerable distance longitudinally in the cord to reach the peripheral nerve from the giant fibre. The giant fibre-supply of the longitudinal musculature thus maintains itself constant even when the giant fibre is absent from a section of the nerve-cord.

The giant fibre branches described above are all efferent branches. A systematic study has not been made of afferent fibres connected with the giant axon. Nevertheless, it has been observed that beside the peripheral branches small fibres pass from the ventral surface of the giant fibre to terminate in the neuropile. The peripheral nerves contain a large number of fine sensory fibres which originate in the epidermis and fan out into the neuropile on entering the nerve-cord, many of them lying just below the giant axon. This histological picture would suggest that there is ample opportunity for the giant fibre to be fired directly by impulses entering the nerve-cord via the sensory nerves, contact between afferent neurones and giant axon occurring either on the surface of the latter, or between fine fibres forming synapses in the neuropile.

### (g) *Effect of cutting the nerve-cord*

The arrangement of the giant fibre and its cells, and the conception that it represents a vast syncytium extending throughout the length of the body and, indeed, throughout the nervous system and the body-wall, have been based on the examination of preserved material only. Arguing on the basis of our knowledge of nerve-fibres in other animals one would expect that the giant fibre of Myxicola would show one of the following arrangements—it could (1) arise from a single large cell at either the anterior or posterior end of the body; (2) arise from a number of large or small cells at either the anterior or posterior end of the body; (3) arise from many small and/or large cells throughout its length. The picture which has been presented is that of a very large fibre which branches and fuses together several times in the anterior region of the body, which arises from two large cells in the supra-oesophageal ganglia, and which is connected with numerous smaller cells throughout its length. To test the validity of this description, the nerve-cord

and its contained giant fibre were transected in the thoracic region of a number of specimens, the animals were killed after periods ranging from 1 to 16 days, and the nerve-cords were fixed in Bouin's fluid or picro-formol and sectioned for microscopic examination.

There are inherent difficulties in such an experiment owing to the fact that *M. infundibulum*, unlike the allied species *M. aesthetica* and *Sabella pavonina* (Okada, 1932, 1934; Berrill and Mees, 1936, 1936a), not only does not regenerate lost tissue, but survives poorly, and begins to show signs of decomposition which spreads from the injured area. In some cases the entire animal was transected in the thorax, and anterior and posterior portions were placed in separate finger-bowls. Heads and thoraces survived well up to at least 30 days, while abdominal portions became completely decomposed after 10 days. The behaviour of the animal provides a useful index of the condition of the giant fibre in injured specimens, since presence or absence of the quick contraction reveals whether or not the axon is healthy. Decomposition affects the giant axon sooner than the rest of the central nervous system. Frequently when the giant fibre response can no longer be elicited, the animal still shows antiperistaltic waves of thickening which extend up to the site of injury, and histological examination of the nerve-cord of such decomposed specimens reveals that the axoplasm of the giant fibre has degenerated into discrete clumps of protein over very considerable stretches, while the remaining neurones of the cord appear normal. This phenomenon can be satisfactorily ascribed to the fact that the giant fibre is a continuous structure and provides an uninterrupted avenue for the transmission of traumatic changes. Nevertheless, it has been possible to obtain several specimens which survived well up to 16 days after section of the nerve-cord, and which showed the giant fibre response throughout that period. In these specimens there was no indication of degeneration of the giant fibre either anterior or posterior to the cut surface. The giant fibre had sealed itself off on either side of the lesion. No regeneration or fusion of the severed ends had occurred; otherwise the fibre, anteriorly and posteriorly, appeared normal.

Information about degeneration times of invertebrate nerve-fibres is scanty, but that which is available indicates that degeneration of the distal portion of an axon separated from its cell-body should occur within 16 days. Although Mönckeberg and Bethe (1899) found that degeneration in the peripheral stump of frog nerves had progressed only a few mm. between 12 and 20 days after injury, Sereni and Young (1932) showed that the course of degeneration in the stellar and mantle nerves of the octopus was very rapid. Granulations had appeared in the axons separated from their cell bodies within 15 hours and after 24 hours the distal axon was full of breakdown products. Also Holmes (1942) found marked degeneration of the smaller fibres in the prawn, 3 days after section of the nerve-cord, although the median giant fibres in the nerve-cord of this animal, fibres which probably are syncytia, failed to degenerate up to 14 days after operation. These experiments indicate that some degeneration of the giant axon of Myxicola should have occurred either

anterior or posterior to the cut region if the fibre has a unicellular origin, or if its nerve-cells are aggregated together in one locus. Since the giant fibre
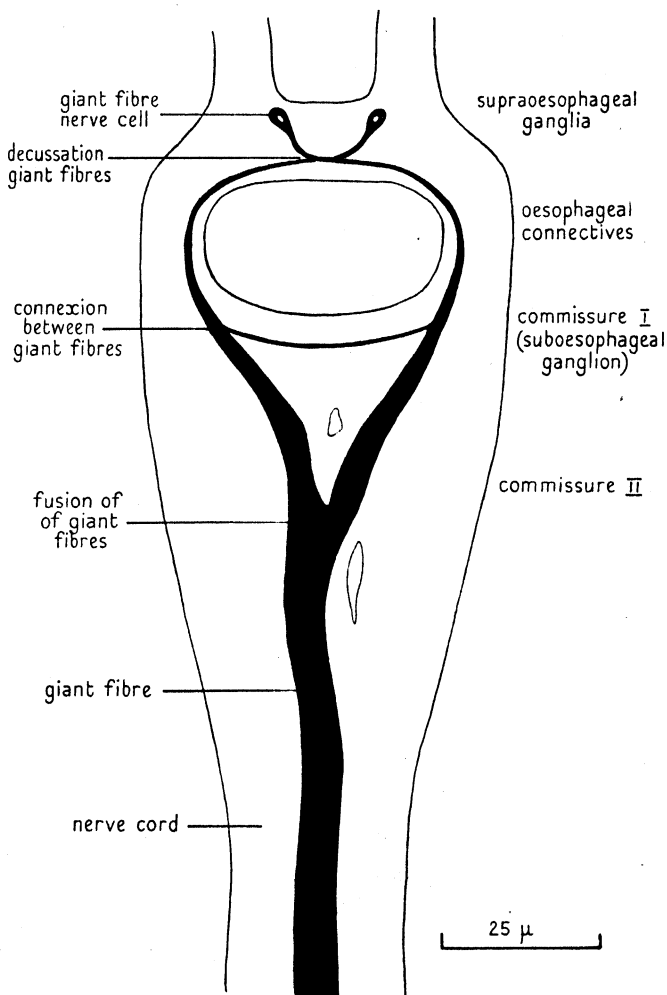


TEXT-FIG. 12. Reconstruction of the central nervous system and giant fibre in the anterior region of *M. aesthetica* (prostomium and setigers I–III). The figure is based on measurements of maximal horizontal diameters in serial transverse segments. Two divisions of the nerve-cord occur in setigers I and II.

is a continuous structure throughout its length the result of this experiment can mean only that it is connected to nerve-cells both anterior and posterior to the thorax. In fact, the giant fibre is a syncytium with nerve-cells throughout its length.

## 2. *M. aesthetica* Claparède

The nervous system of this species resembles rather closely that of *M. infundibulum*. In general configuration it consists of a short'and broad mass of supra-oesophageal ganglia in the prostomium, oesophageal connectives arising from the posterior and ventral surface of these ganglia and joining the sub-oesophageal ganglion in setiger I, and a largely unpaired nerve-cord which extends to the extreme posterior end of the animal (Text-fig. 12; Pl. 3, figs. 17 and 18). The nerve-cord lies in the longitudinal muscle layer and forms a broad flattened mass extending from the sub-epidermal connective
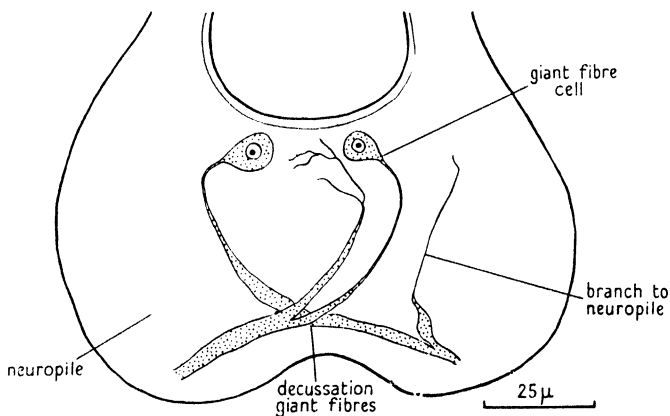


TEXT-FIG. 13. Reconstruction of the two giant fibres and their nerve-cells in the supra-oesophageal ganglia of *M. aesthetica*, based on camera lucida drawings. The outline of the neuropil only is shown; the other nerve-cells of the supra-oesophageal ganglia lie around this region. The nerve-cells of the giant fibres lie slightly ventral as well as anterior to the latter.

tissue to the coelomic cavity. It is attached laterally to the muscular layer, and dorsally to the ventral mesentery which joins the gut above and which contains a relatively large ventral blood-vessel and a bundle of chloragogen cells. In a specimen 11 mm. long the supra-oesophageal ganglia were $250\mu$ wide, the oesophageal connectives $50\mu$, sub-oesophageal ganglion $220\mu$, and the nerve-cord in setiger II $130\mu$ wide. Posteriorly the cord tapers off. It is about $100\mu$ wide in the posterior thorax, diminishes in the posterior abdomen to about $15\mu$, and gradually expands again in the last few segments to reach a diameter of about $30\mu$ at the posterior end. Although the nerve-cord forms a single strand throughout most of its length, it is bipartite in the anterior thorax as in the larger species of this genus. It divides once in the posterior end of setiger I and again in setiger II, forming a double cord over short intervals of less than one segment in extent. It is covered externally by a thin sheath of connective tissue, overlain by peritoneum on its dorsal face. Nerve-cells are distributed along the entire length of the nerve-cord,

forming a peripheral layer and almost completely investing it, except for a medio-dorsal interval. The interior of the cord contains the neuropile, supporting fibres and cells and, in the superior median region, the giant fibre (Text-fig. 14A).

The giant fibre begins in the supra-oesophageal ganglia. A decussation of two fibres occurs in the posterior and inferior portion of these ganglia, as in *M. infundibulum*, and, after crossing the median sagittal plane, the fibre passes down the contralateral oesophageal connective (Text-figs. 13 and 14B). The two fibres fuse together in the posterior portion of setiger I in the second commissure and the single fibre then extends to the posterior end in the mid-dorsal line. It is relatively smaller than in *M. infundibulum*, when compared with the total volume of the nerve-cord. It has a diameter of about $5\mu$ in the supra-oesophageal ganglia, and increases to $10\mu$ in the oesophageal connectives. It is $40\mu$ broad after fusion in the cord behind the sub-oesophageal ganglion and diminishes gradually to $20\mu$ in the posterior thorax. In the abdomen its diameter decreases from $10\mu$ anteriorly to $2\mu$ posteriorly, with a slight resurgence to $5\mu$ in the posterior swelling of the nerve-cord before terminating in a fine thread at the extreme posterior end. The cord shows regular, segmental variations in diameter, swelling in the middle of each segment and diminishing at intersegmental levels, but although the giant fibre shows some variations in diameter from place to place apart from the trend towards diminution posteriorly, such variations of the giant fibre seem to bear no definite relation to alterations in volume of the nerve-cord itself.

The giant fibre appears to be homogeneous in transverse and longitudinal sections. There is no clearly marked sheath: the fine fibrils which surround the fibre form part of the general supporting network of the cord and show no particular concentration nor peculiarity of arrangement in this area.

Horizontal longitudinal sections of the supra-oesophageal ganglia show that on each side of the region where the giant fibres decussate in the mid-line several branches are given off laterally or anteriorly into the neuropile. Only one of these branches, on each side, has been followed conclusively to its termination and it has been found that it curves anteriorly and ventrally to terminate in a relatively large cell lying centrally on the ventral face of the ganglia. These two large giant fibre cells are symmetrically disposed, one on each side of the mid-line, and are unipolar. They are about $12\mu \times 16\mu$ in diameter; the nucleus is $8\mu \times 10\mu$. The cytoplasm is somewhat basiphilic, but stains feebly in all the methods employed, and contains one or a few vacuoles. The nucleus encloses a single nucleolus, $2\mu$ in diameter, and smaller flakes of chromatin. The arrangement of the two giant fibres and their cells in the supra-oesophageal ganglia is very similar, therefore, to that which occurs in *M. infundibulum*. The only significant point of difference is that the giant fibre-cells lie much farther anterior in the supra-oesophageal ganglia of *M. aesthetica* and the giant fibres must extend a long distance anteriorly in the neuropile before reaching them (Text-figs. 13 and 14B).
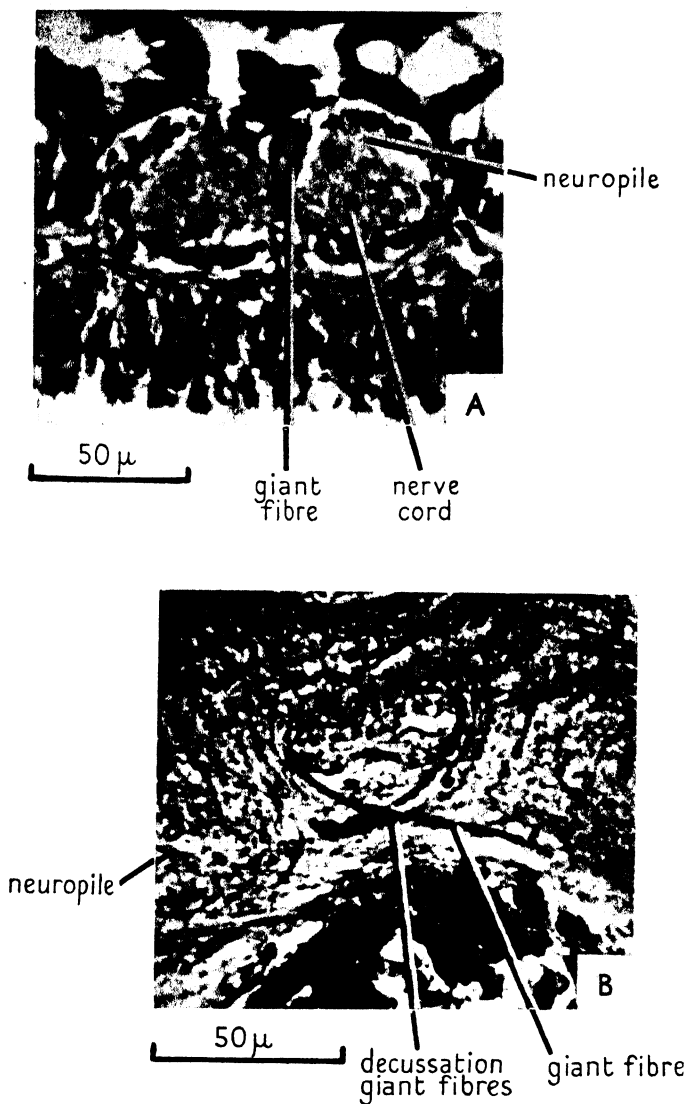
TEXT-FIG. 14. Photographs of sections of *M. aesthetica*. Picro-formol, cedarwood oil, paraffin, Holmes's silver. 14A. Transverse section of the nerve-cord in the posterior thoracic region. 14B. Horizontal longitudinal section through the level of decussation of the giant fibres in the supra-oesophageal ganglia.

Nerve-cells in the cord stain very poorly with routine stains, such as haematoxylin, and are only slightly impregnated by silver. They vary in size from $8\mu \times 10\mu$ to $12\mu \times 15\mu$. The nuclei range from $4\mu \times 7\mu$ to $8\mu \times 10\mu$. The cytoplasm is finely granular and basiphilic and contains several large vacuoles. The nucleus has a rather scanty chromatin content, and a single nucleolus, $2\mu$ in diameter.

Nerve-fibres in the cord are very small, less than $0.5\mu$ in diameter, and this fact, coupled with the weak staining characteristics of the nerve-cells themselves, has rendered it very difficult to determine the cellular connexions of the giant fibre. Nerve-cells at frequent intervals were seen to send a single process towards the giant fibre, but in no case was such a connexion established with certainty. No branches of the giant fibres were seen, although they certainly exist in some form.

## VI. Discussion

The giant fibres of Myxicola are unique, even among the Polychaeta in which so many species possess these structures, by virtue of their large size. The maximal diameter of about 1 mm. which the axon occasionally attains in the thoracic region is not exceeded in the animal kingdom, and is equalled only by the giant axons of the stellar nerves of Loligo (Young, 1934, 1936). Comparable fibres of extraordinarily large size relative to that of the animal have been described in the Oligochaete, *Branchiura sowerbyi* Beddard (Stephenson, 1912). Friedländer (1894) had already suggested that the sheath of the giant fibre of *M. infundibulum* was slightly myelinated, and this fact is confirmed by the use of Sudan black which reveals a slight concentration of lipoid about the axon. Among those Invertebrates which possess giant axons there is a great deal of variation in the presence or absence of myelin about these structures. Friedländer (1889), for example, was able to show that the giant fibres of the Annelids Lumbricus and Mastobranchus possessed conspicuous myelin sheaths which he revealed both by treatment with $OsO_4$ and by examination in polarized light. Ashworth (1909) and Gamble and Ashworth (1900) failed to find a myelin layer about the giant fibres of Halla and Arenicola, respectively, that reduced osmium tetroxide. When the sheath contains only a small amount of lipoid, however, osmium tetroxide is quite ineffective in revealing its presence, as recent studies making use of polarized light have shown. Göthlin (1913) showed that nerve-sheaths which do not normally reveal birefringence due to lipoids may be made to do so by immersion in liquids of suitable refractive index. Bear, Schmitt, and Young (1937) have demonstrated the existence of such a metatropic sheath, that does not stain with osmium tetroxide, in a layer about $4\mu$ thick surrounding the giant axons of the squid. More recently, Taylor (1940) has shown in the earthworm that the sheath of the giant fibres, normally myelotropic and negatively birefringent with regard to its length, may become metatropic at fibre diameters of $10\mu–12\mu$. In *M. infundibulum* the fatty sheath appears to resemble the metatropic sheath of squid axons, except that there is no internal cellular

layer separating it from the axon itself, as occurs in Cephalopods and Crustacea (Bear, *et al.*, 1937). Further studies making use of polarized light should be very informative.

Pumphrey and Young (1938) have calculated that in the case of the squid, possession of giant axons results in a significant saving of conduction time, a factor of obvious survival value when considered in the light of the quick flight reaction which they mediate, and it is interesting to apply the same argument to Myxicola. In this species the fibre is concerned with a mechanism of quick contraction which causes the animal to retract swiftly into its tube. Ordinary peristaltic waves of thickening, involving successive neurones, are quite slow in their course along the body of this species, and require a few seconds to traverse the length of the animal. The very fact that the final common pathway involved in this quick contraction is a single axon continuous throughout the nerve-cord and with branches penetrating the entire body-wall obviates all the synaptic delay involved in interneuronal transmission, in the efferent side of the nervous pathway at least. Nicol and Whitteridge (unpublished) have found that the velocity of the nervous impulse in the giant axon of Myxicola is about 11 m. per sec. in the anterior half of an extended animal, a figure that can be taken as a minimal estimation of conduction speed. In the case of Aphrodite, where giant fibres are absent (Cunningham, 1888; Rohde, 1887), Jenkins and Carlson (1903) found a nervous impulse to be conducted with a velocity of 54·5 cm. per sec. If these two figures are employed for purposes of comparison, and only the anterior 7 cm. of animal is considered, a length sufficient to permit withdrawal into its tube, it follows that possession of a giant axon results in a saving of 87 msec. when an impulse traverses this region. The size of the axon is therefore clearly of importance in permitting the animal to retract faster. It has long been known that nerves with thick myelin sheaths conduct faster, other factors being equal, than those without, and recently Sanders and Whitteridge (1946) have been able to substantiate this conclusion quantitatively by taking advantage of the fact that in regeneration of cut mammalian nerves the fibres of the proximal stump have thicker myelin sheaths and smaller axon diameters than normal nerves. Myxicola is obviously another example among the Invertebrates where increase of conduction time has resulted from increase in fibre diameter solely, without concomitant increase in myelin sheath thickness.

Giant nerve-fibres have been described in other Sabellids and there is general agreement that they are particularly well developed in this family, and that they occur in all species (McIntosh, 1877, 1885, 1923; *et al.*). They consist, in general, of very large fibres which extend the entire length of the animal. Two separate patterns can be distinguished, however, which are related to the configuration of the nerve-cord. In the Sabellinae and Fabriciinae, the nerve-cord is double throughout and conforms to the classical 'ladder' pattern, and a large nerve-fibre lies in each half of the nerve-cord. The two fibres are connected together in the commissures of the thoracic

region at least, and extend through the oesophageal connectives into the supra-oesophageal ganglia (Sabella, Branchiomma, Euchone; Claparède, 1873; de St. Joseph, 1894; Brunotte, 1888; Evenkamp, 1931; Thomas, 1940; *et al.*). In the Myxicolinae, on the other hand, a considerable unification of the cord has occurred and this has extended to the giant fibres. The nerve-cord is double for short stretches in the anterior thoracic region only, and is single posterior to that region. In conformity with this arrangement there are two giant fibres in the first few setigers, where they interconnect by transverse anastomoses as in Sabella, while in the posterior thorax and abdomen the fibre is single. The giant fibre also extends into the supra-oesophageal ganglia in this sub-family.

The existence of syncytial giant fibres among different Invertebrates is now a well-recognized phenomenon and Myxicola forms an example at one end of a graded series of such structures which can be traced among the Annelida. At the one extreme there are the giant axons of Halla and Aglaurides, which are unicellular and which extend through a large part of the nerve-cord (Ashworth, 1909). Among the Aphroditids, the giant fibres may be unicellular or a single fibre may arise from the fusion of the processes of a few nerve-cells (Rohde, 1887). The fibres are completely multicellular among the Arenicolidae, where they form an anastomosing syncytium connected with nerve-cells in each segment (Gamble and Ashworth, 1900). Similarly, in the Oligochaeta, some of the giant fibres may anastomose with one another, and their nerve-cells are arranged along the length of the nerve-cord (Friedländer, 1888; Stough, 1926). Stough (loc. cit.) has described transverse, segmentally arranged septa in the giant fibres of both Lumbricids and Nereis. Finally, at the other extreme of this series, lies Myxicola in which the giant fibre is a huge syncytium, continuous throughout its length in the central nervous system, without internal dividing septa, and connected with nerve-cells throughout its entire course.

It is obvious that structures as large as these fibres, and which occupy such a large proportion of the central nervous system, must play an important role in the life of the animal. The most noteworthy feature of the Sabellids is their sedentary mode of existence. All species are tubicolous and retract quickly into their tubes when properly stimulated. This is a mass reaction involving all the longitudinal musculature. Friedländer (1889) stated that Myxicola showed little activity apart from the quick contraction, but this is by no means the only reaction performed by this animal. It also shows slow oscillatory movements leading to burrowing and peristaltic and antiperistaltic waves after suitable stimulation (Nicol, unpublished). Such reactions imply, of course, the existence of a definite, metameric pattern of neurones. They are, however, relatively simple, and there is none of the more complex behavioural patterns such as the sinuous lateral swimming movements which occur in Nereis. It is reasonable to conclude, therefore, that the adoption of a sedentary mode of existence, with attendant abolition of the need for the more complex patterns of behaviour characteristic of free-living forms, has per-

mitted one part of the central nervous system to become highly hypertrophied along with modification of other structural features for a sedentary habit. The resultant modification is a huge, syncytial axon, which causes the animal to act as a unit in quick withdrawal from noxious stimuli. Specialization has led to great simplification of structure and response, but it has also led to restriction in the range of responses possible and, therefore, to sacrifice of plasticity in behaviour patterns.

### VII. Summary

1. A description is given of the main features of the central nervous system of *Myxicola infundibulum* Rénier.

2. The nerve-cord is double in the first four thoracic segments and single posteriorly. It shows segmental swellings but is not ganglionated in the usual sense in that nerve-cell accumulations are not related directly to such swellings of the cord.

3. A very large axon lies within the dorsal portion of the nerve-cord and extends from the supra-oesophageal ganglia to the posterior end of the animal. It is small in the head ganglia where it passes transversely across the mid-line, increases in diameter in the oesophageal connectives, and expands to very large size, up to 1 mm., in the posterior thorax and anterior abdomen, and gradually tapers off to about $100\mu$ in the posterior body. It shows segmental swellings corresponding to those of the nerve-cord in each segment. It occupies about 27 per cent. of the volume of the central nervous system and 0·3 per cent. of the volume of the animal. The diameter of the fibre increases during contraction of the worm.

4. The giant fibre is a continuous structure throughout its length, without internal dividing membranes or septa. Usually a branch of the giant fibre lies in each half of the nerve-cord in the anterior thoracic segments and these several branches are continuous with one another longitudinally and transversely.

5. The giant fibre is connected with nerve-cells along its entire course; it arises from a pair of cells in the supra-oesophageal ganglia, and receives the processes of many nerve-cells in each segment. There is no difference between the nerve-cells of the giant fibre and the other nerve-cells of the cord.

6. A distinct fibrous sheath invests the giant fibre. A slight concentration of lipoid can be revealed in this sheath by the use of Sudan black.

7. About eight peripheral branches arise from the giant fibre in each segment. They have a complex course in the nerve-cord where they anastomose with one another and receive the processes of nerve-cells. Peripherally, they are distributed to the longitudinal musculature.

8. Specimens surviving 16 days following section of the nerve-cord in the thorax have shown that the giant fibre does not degenerate in front of or behind a cut, thus confirming that it is a multicellular structure connected to nerve-cells in the thorax and abdomen.

9. It is concluded that the giant fibre of *M. infundibulum* is a large syncytial structure, extending throughout the entire central nervous system and the body-wall of the animal.

10. The giant fibre system of *M. aesthetica* resembles that of *M. infundibulum*.

11. Some implications of the possession of such a giant axon are discussed. It is suggested that its size, structure, and simplicity lead to rapid conduction and thus effect a considerable saving of reaction time, of considerable value to the species when considered in the light of the quick contraction which it mediates. The adoption of a sedentary mode of existence has permitted this portion of the central nervous system to become developed at the expense of other elements concerned with errant habits.

## REFERENCES

ASHWORTH, J. H., 1909. Phil. Trans. Roy. Soc. B, **200,** 427.
BAKER, J. R., 1944. Quart. J. micr. Sci., **85,** 1.
—— 1946. Ibid., **87,** 441.
BEAR, R. S., SCHMITT, F. O., and YOUNG, J. Z., 1937. Proc. Roy. Soc. B, **123,** 496 and 505.
BERRILL, N. J., and MEES, D., 1936. J. exp. Zool., **73,** 67.
—— 1936a. Ibid., **74,** 61.
BODIAN, D., 1936. Anat. Rec., **65,** 89.
—— 1937. Ibid., **69,** 153.
—— 1937a. J. comp. Neurol., **68,** 117.
—— 1940. Ibid., **73,** 323.
BOULE, L., 1908. Le Névraxe, **9,** 313.
—— 1909. Ibid., **10,** 13.
BOVARD, J. F., 1918. Univ. Calif. Publ. Zool., **18,** 135.
BRUNOTTE, C., 1888. Trav. Sta. Zool. Cette. Quoted by Ashworth (1909).
BULLOCK, T. H., 1944. J. comp. Neurol., **80,** 355.
—— 1945. J. Neurophysiol., **8,** 55.
—— 1945a. Quart. J. micr. Sci., **86,** 55.
CARLETON, H. M., 1922. Quart. J. micr. Sci., **66,** 501.
CLAPARÈDE, E., 1861. Mém. Soc. Phys. Hist. Nat. Genève, **16,** 71.
—— 1870. Ibid., **20,** 365.
—— 1873. Ibid., **22,** 1.
COLE, E. C., 1946. Stain Tech., **21,** 63.
CUNNINGHAM, J. T., 1888. Ibid., **28,** 239.
DEMPSEY, E. W., and WISLOCKI, G. B., 1946. Physiol. Rev., **26,** 1.
ECCLES, J. C., GRANIT, R., and YOUNG, J. Z., 1933. Proc. Physiol. Soc. in J. Physiol., **77,** 23P.
EISIG, H., 1887. Fauna u. Flora Neapel, Monogr., **16,** xxvii+906.
EVENKAMP, H., 1931. Zool. J. Abt. Anat., **53,** 405.
FEDOROW, B., 1928. Jb. Morph. mikr. Anat., **12,** 347.
FOLEY, J. O., HUNT, E. A., and SACKETT, W. W., Jr., 1936. Anat. Rec., **64** (Suppl. 3), 65.
FRIEDLÄNDER, B., 1888. Z. wiss. Zool., **47,** 47.

FRIEDLÄNDER, B., 1889. Mitt. Zool. Sta. Neapel, **9**, 205.
—— 1894. Z. wiss. Zool., **58**, 661.
GAMBLE, F. W., and ASHWORTH, J. H., 1900. Quart. J. micr. Sci., **45**, 419.
GÖTHLIN, E. F., 1913. K. svenska Vetenskak. Handl., **51**, 1. Quoted by Schmitt (1936).
HAMAKER, J. I., 1898. Bull. Mus. Comp. Zool. Harvard, **32**, 89.
HANSTRÖM, B., 1928. *Vergleichende Anatomie des Nervensystems der wirbellosen Tiere.* Berlin.
HOLMES, W., 1942. Phil. Trans. Roy. Soc. B, **231**, 293.
—— 1947. *Axon Stain,* in *Recent Advances in Clinical Pathology,* General Editor, S. C. Dyke. London.
JENKINS, O. P., and CARLSON, A. J., 1903. J. comp. Neurol., **13**, 259.
JOHANSSON, K. E., 1927. Zool. Bidrag, **11**, 1.
KOWALSKI, J., 1909. La Cellule, **25**, 289.
KRAWANY, J., 1905. Arb. Zool. Inst. Wien, **15**, 281.
LEWIS, M., 1898. Proc. Amer. Acad. Arts Sci., **33**, 225.
LISON, L., 1936. *Histochimie animale.* Paris.
McINTOSH, W. C., 1877. Proc. Roy. Soc. Edinb., **9**, 372 (Abstract).
—— 1885. Challenger Reports, **12**, xi + 554.
—— 1908–23. *A Monograph of the British Annelids,* Vols. 2, 3, 4. London (Ray Society).
MAXIMOV, A. A., and BLOOM, W., 1944. *A Text-book of Histology,* Fourth Edition. Philadelphia and London.
MEYER, E., 1887. Mitt. Zool. Sta. Neapel, **7**, 592.
—— 1888. Ibid., **8**, 462.
MÖNCKEBERG, G., and BETHE, A., 1899. Arch. mikr. Anat., **54**, 135.
MONTAGU, G., 1808. Trans. Linn. Soc., **9**, 81.
NANSEN, F., 1887. Bergens Mus. Aarsb., 1886, p. 27.
NICOL, J. A. C., and YOUNG, J. Z., 1946. Nature, **158**, 167.
OKADA, Yô K., 1932. Annot. Zool. Jap., **13**, 535.
—— 1934. Bull. Sc. Fr. Belg., **68**, 340.
OWENS, H. B., and BENSLEY, R. R., 1929. Amer. J. Anat., **44**, 79.
PANTIN, C. F. A., 1946. *Notes on Microscopical Technique for Zoologists.* Cambridge.
PRUVOT, G., 1885. Arch. Zool. exp. gén., **3**, 211.
PUMPHREY, R. J., and YOUNG, J. Z., 1938. J. exp. Biol., **15**, 453.
RAMÓN Y CAJAL, S., 1904. Trab. Lab. Invest. Biol. Univ. Madrid, **3**, 277.
RETZIUS, G., 1891. Biol. Unter., Neue Folge, II, 1.
—— 1892. Ibid., III, 1.
ROHDE, E., 1887. Zool. Beiträge, **2**, 1.
RUSHTON, W. A. H., 1945. Proc. Roy. Soc. B, **132**, 423.
—— 1946. Ibid., **133**, 109.
DE ST. JOSEPH, BARON, 1894. Ann. Sci. nat. Zool., **17**, 1.
SANDERS, F. K., and WHITTERIDGE, D., 1946. J. Physiol., **105**, 152.
SCHMITT, F. O., 1936. Cold Spring Harbour Symposium Quant. Biol., **4**, 7.
SERENI, E., and YOUNG, J. Z., 1932. Publ. Staz. Zool. Napoli, **12**, 173.
STEPHENSON, G., 1912. Trans. Roy. Soc. Edinb., **48**, 285.
—— 1930. *The Oligochaeta.* Oxford.
STOUGH, H. B., 1926. J. comp. Neurol., **40**, 409.
—— 1930. Ibid., **50**, 217.
TAYLOR, G. W., 1940. J. cell. comp. Physiol., **15**, 363.
TEN CATE, J., 1938. Arch. Néerland. Physiol., **23**, 136.
THOMAS, J. G., 1940. *Pomatoceros, Sabella and Amphitrite.* L.M.B.C. Mem., **13.**
WAWRZIK, E., 1892. Zool. Beiträge, **3**, 107.
WILSON, D. P., 1936. Quart. J. micr. Sci., **78**, 543.
YOLTON, L. W., 1923. Proc. Nat. Acad. Arts Sci., **9**, 383.
YOUNG, J. Z., 1934. Proc. Physiol. Soc. in J. Physiol., **83**, Pl.
—— 1935. Nature, **135**, 823.
—— 1936. Proc. Roy. Soc. B, **121**, 319.
—— 1938. J. exp. Biol., **15**, 170.
—— 1939. Phil. Trans. Roy. Soc. B, **229**, 465.
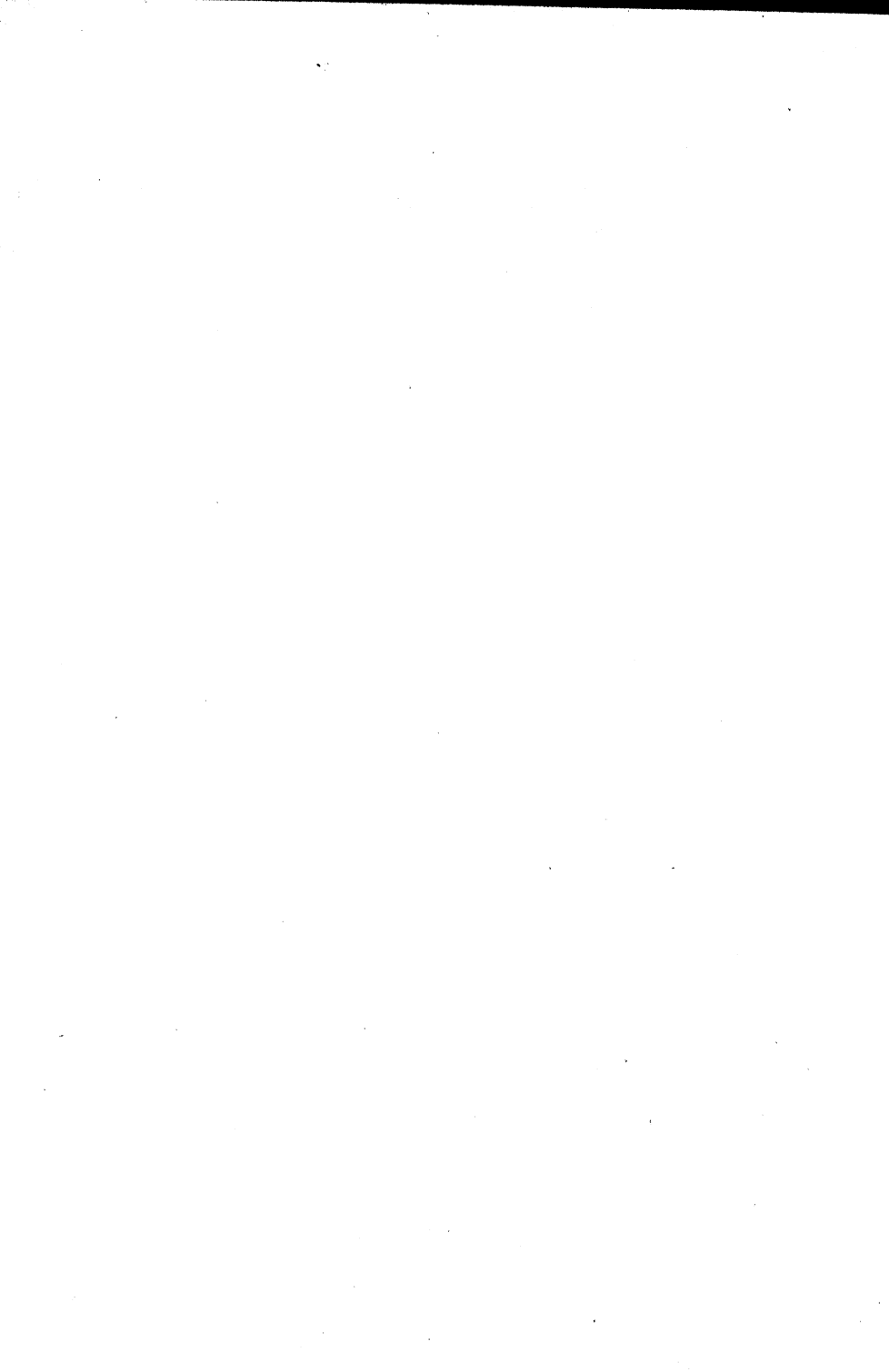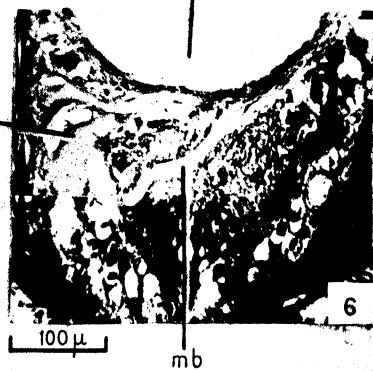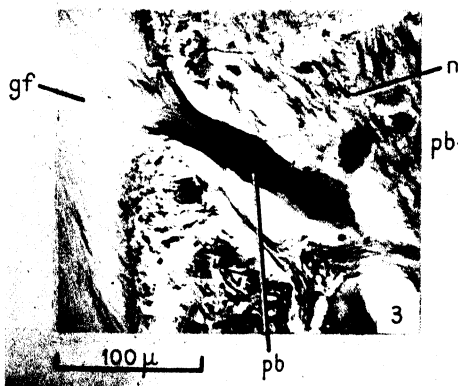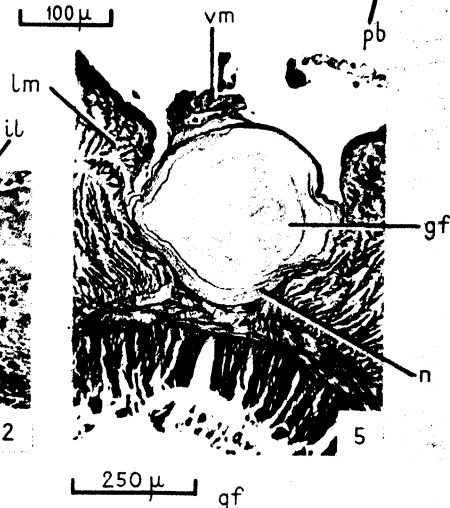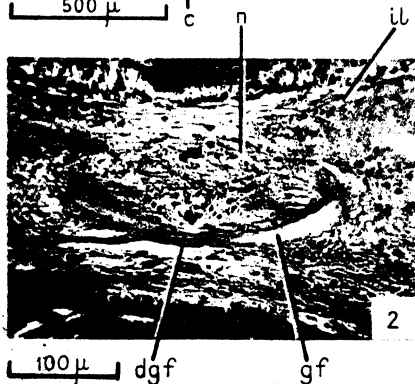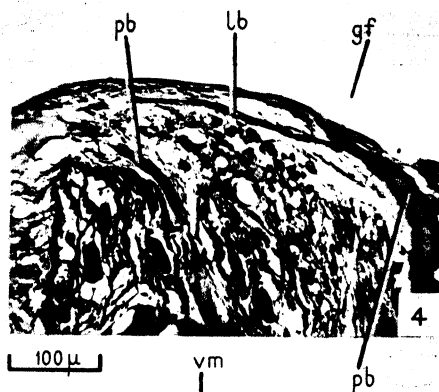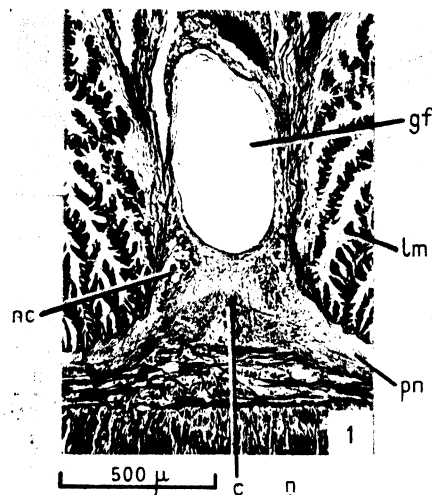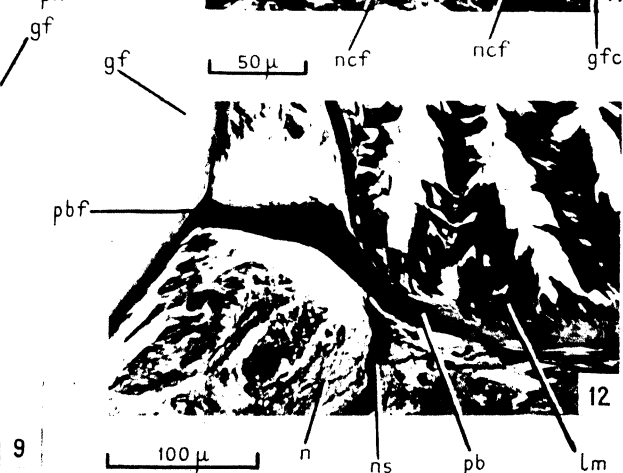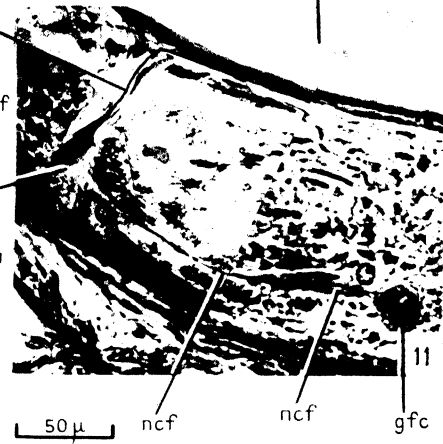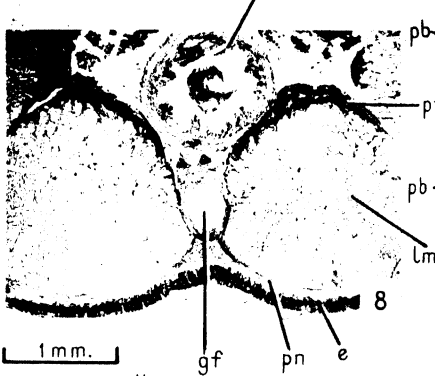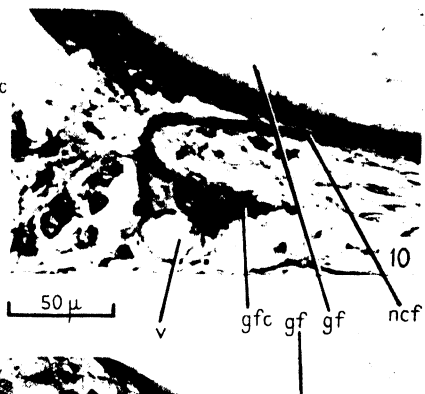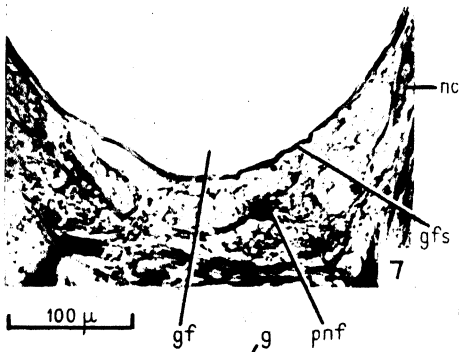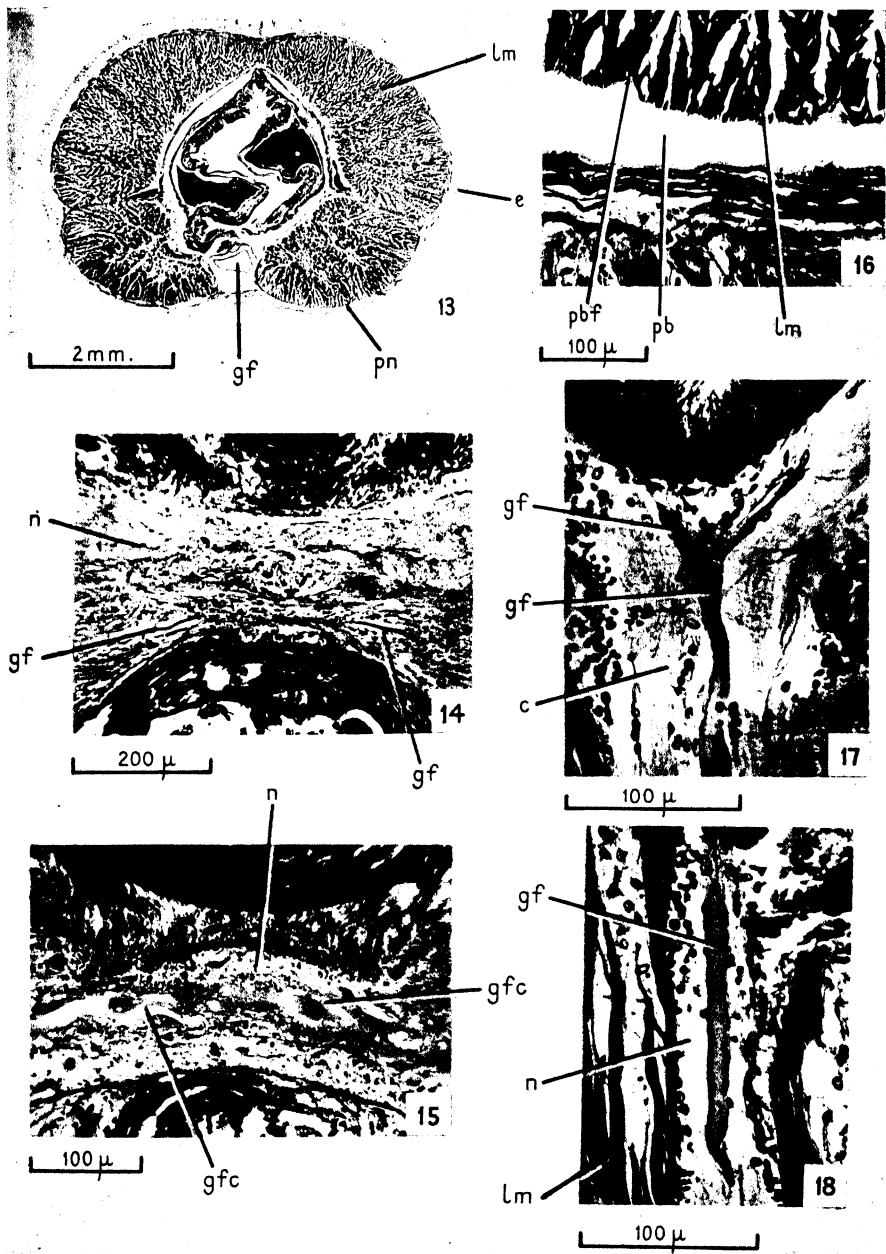
# Transport of Food through the Alimentary Canals of Aquatic Annelids

BY

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DURING an investigation of the blood system in polychaetes of the families Serpulidae and Sabellidae the musculature of the alimentary canal has been studied. In the following species the only muscle coat, which consists of fibres lying transverse to the main axis, lies outside the blood sinus which envelops the canal: *Serpula vermicularis* L., *Hydroides norvegica* (Gunnerus), *Vermiliopsis infundibulum* (Philippi), *Pomatoceros triqueter* L., *Protula intestinum* (Lamarck), *Apomatus ampulliferus* Philippi, *Salmacina incrustans* Claparède, *Spirorbis militaris* (Claparède), *S. corrugatus* (Montagu), *Potamilla* sp. and *Dasychone lucullana* (Delle Chiaje). In *Sabella spallanzanii* (Viviani) (Ewer, 1946) there is another layer of circular muscles between the sinus and the gut epithelium. This inner muscle coat has also been seen by Evenkamp (1931) in *Laonome kroyeri* and *Euchone papillosa*, and Dr. A. Stock (personal communication) has found it in *Sabella pavonina*. In the rest of the literature on serpulids and sabellids the only circular muscle coat recorded is the one situated in the outer wall of the blood sinus. It has been known for a long time (Claparède, 1873) that these muscles contract antiperistaltically, moving the blood forwards in the sinus. In the smaller species it can be seen that the antiperistaltic contraction waves not only constrict the sinus but frequently may also slightly constrict the lumen of the gut. When a contraction wave passes a food bolus the latter is moved for a short distance towards the mouth, but after the passage of the wave quickly returns to its original position. Stephenson (1913) in his well-known paper 'On Intestinal Respiration in Annelids' has shown that antiperistaltic contraction of the gut musculature is not a peculiarity of serpulids and sabellids, but, with very few exceptions, is found in all the many aquatic annelids he examined, whether or not they possess a gut sinus. Where a sinus is absent he postulated that the antiperistaltic mode of contraction of the gut musculature has been retained during evolution from ancestors possessing a sinus. Hence, in aquatic annelids in general, the contents of the alimentary canal are not moved along by peristaltic contractions of its wall as they are in other animals, but the activity of the musculature actually tends to hinder their movement towards the anus. The direction of the contraction waves in the extra muscle coat of *Sabella*, *Laonome*, and *Euchone* is not known.

The gut epithelium of aquatic annelids is ciliated, and it is natural to suppose that the cilia are responsible for food transport while the muscles, in

species with a sinus, have a circulatory function. However, Stephenson has stated that, with few exceptions, the cilia in the posterior part of the alimentary canal beat in a postero-anterior direction; and in some species he observed this postero-anterior beating for a long distance in front of the anus. Nevertheless, food passes down the gut and faeces leave the anus. Stephenson was especially interested in the function of this 'ascending ciliary current'. He believed it to be respiratory. He did not demonstrate or discuss at any length how the gut contents move against the current. However, in his account of observations on the oligochaete *Aeolosoma hemprichi* he put forward an hypothesis that could be a plausible general explanation of the mechanism of food transport in all annelids with antiperistaltic muscle contraction and cilia beating towards the mouth. He suggested that the cilia move only the peripheral fluid in the gut and that this ascending peripheral current has as its complement a descending axial current, 'since it is not to be supposed that the postero-anterior current passes through the whole length of the animal and out at the mouth'.

Recently Lindroth (1938) has made some observations which throw doubt upon the existence of an ascending ciliary current. He has pointed out that Stephenson did not demonstrate convincingly that the cilia beat towards the mouth. When one examines the abdominal gut of a small intact annelid one can see that the metachronal waves of the ciliated epithelium travel in a postero-anterior direction. To Stephenson's list of serpulids and sabellids in which this occurs I can add: *Serpula vermicularis, S. lo biancoi* Rioja, *Hydroides norvegica, Vermiliopsis infundibulum, Apomatus ampulliferus, Salmacina incrustans, Spirorbis corrugatus, Amphiglena mediterranea* (Leydig), and *Jasmineira candela* (Grube). However, it is well known (Gray, 1928) that in many animals the direction of ciliary beat may be the opposite of the direction taken by the metachronal waves, for example, in ctenophores. As Lindroth has pointed out, Stephenson did not discriminate between the metachronal waves and the beating of the cilia. He stated only that: 'The direction of action of the cilia is obviously . . . from behind forwards.' Now it is very difficult to discern the direction of the effective strokes of the cilia in the alimentary canals of serpulids and sabellids: they beat too quickly. In intact specimens one cannot deduce the direction of ciliary beat from the direction of movement of particles travelling over the surface of the epithelium because, as already discussed, the contents of the gut may be moving against the current set up by the cilia. Like Stephenson and Lindroth I have noticed that small particles in the water in the vicinity of the anus may enter the rectum and travel up it for a short distance, but they are invariably expelled. Stephenson thought the entrance of particles into the rectum to be additional evidence for the existence of an ascending ciliary current; he attributed the expulsion of the particles to an inability on the part of the posterior end of the gut 'to deal with solid particles'; 'if possible it avoids receiving them'. Lindroth has shown that the particles are sucked into the rectum during the expansion of the rectum which follows the initiation of a wave of antiperistaltic contraction of its walls.

Lindroth has seen the direction of beating of individual cilia in the posterior part of the alimentary canal of whole small specimens of *Ammotrypane aulogaster* (Opheliidae). They were beating towards the anus. Moreover, particles near the cilia moved towards the anus, as they also did in the hesionids *Ophiodromus vittatus* and *Castalia punctata* and in an unidentified member of the same family. Lindroth did not consider the possibility that these particles might have been moving under the influence of an axial stream of water returning down the gut. His observations were all made on animals immobilized by narcotization with chloretone; although I know of no evidence that chloretone affects the beating of cilia, it would be desirable to make observations on normal animals. I have found it impossible to distinguish the direction of beating of the cilia in the intact alimentary canals of any of the serpulids and sabellids I have examined. *Salmacina incrustans*, being relatively very transparent, might be thought suitable for this purpose, but the cilia beat too quickly. Preliminary attempts to slow down their rate of movement by lowering the temperature or pH of the surrounding water were unsuccessful. Even when specimens are dying by compression and asphyxiation under a coverslip the cilia beat rapidly up to the moment when their movement entirely ceases.

In larger serpulids and sabellids one can dissect out portions of the wall of the abdominal alimentary canal and study the direction of movement of particles over the exposed ciliated surface. The interpretation of observations is then not complicated by the possibility that the particles are moving against the current set up by the cilia. In this way it has been found that in the following species the cilia beat towards the anus whilst the metachronal waves travel towards the mouth: *Serpula vermicularis*, *Hydroides norvegica*, *Vermiliopsis infundibulum*, *Pomatoceros triqueter*, *Protula intestinum*, and *Sabella spallanzanii*.

Hence it is clear that the cilia in the alimentary canals of these serpulids and sabellids beat in an antero-posterior direction, and that the 'ascending ciliary current' of Stephenson does not exist. Moreover, it seems improbable that it exists in any aquatic annelids. Lindroth (1941) has already discussed the respiration of polychaetes and concluded that the rectum is not a special respiratory organ as Stephenson thought it to be.

In conclusion, it is very probable that the cilia of the alimentary canal in aquatic annelids directly aid transport of its contents. The force exerted by the cilia may be greater than that exerted by the antiperistaltically contracting muscles, but it is probable that food transport is aided by a descending stream of gut fluid initiated in the following manner at the anterior end of the gut. When the mouth is closed or when food is entering the mouth in the course of ciliary feeding, the fluid moved forwards by the antiperistaltic contraction waves is presumably forced to turn back and move down the gut towards the anus. In the same way Stephenson accounted for the transport of food in the gut of *Aeolosoma* in the face of a peripheral antero-posterior stream of water.

The metachronal waves on the ciliated epithelium of the intestine of *Salmacina incrustans* move in a postero-anterior direction, but in the wide part of the alimentary canal situated in the achaetous zone just behind the thorax the waves travel obliquely backwards, the transverse component always appearing clockwise when the animal is viewed from the anterior end. Under the influence of these cilia the boli of mucus and food particles rotate as they move down the intestine and continue to rotate until they have left the anus. A faecal bolus is sometimes arrested half inside and half outside the anus; both parts continue to rotate. In one case a bolus in the rectum was observed rotating while all the cilia in that part of the gut were inactive. The rotation imparts a characteristic spirally sculptured shape to the bolus, and the region of greatest concentration of particles within it takes the form of a spiral. These boli invariably rotate in a direction that appears clockwise when the animal is viewed from the anterior end. This shows that the cilia causing the rotation beat in the same direction as that taken by the metachronal waves. On the contrary, as discussed above, the cilia of the intestine most probably beat in a direction opposite to that taken by the metachronal waves.

Boli rotating in a clockwise direction have also been seen in the abdominal gut of *Serpula vermicularis*, *Pomatoceros triqueter*, and *Spirorbis corrugatus*. Spirally shaped boli have been found in the abdominal gut in *Protula intestinum*, *Sabella spallanzanii*, *Potamilla* sp., and *Dasychone lucullana*. Stephenson (1913) noticed a rotating bolus in the posterior part of the alimentary canal of a specimen of *Spirorbis borealis* and he also found rotating boli in *Aeolosoma hemprichi*, an observation that I have confirmed. Faulkner (1930) noticed occasional rotating food masses in the anterior part of the gut of *Filograna implexa*. It seems very probable that they will be found in other annelids. The rotation presumably facilitates the mixing of enzymes with the food as in *Amphioxus* (Barrington, 1937) and slows up the movement of food through the anterior part of the gut which is probably the region where enzymes are secreted (Nicol, 1930).

### SUMMARY

1. In most serpulids and sabellids the only muscle coat in the wall of the alimentary canal lies outside the blood sinus which envelops it. In a few sabellids there is another muscle coat, of unknown function, between the sinus and the gut epithelium.

2. The muscles outside the sinus contract antiperistaltically and tend to hinder the transport of the gut contents towards the anus.

3. The contents of the alimentary canal are transported by its cilia which beat towards the anus. The metachronal waves of the ciliated epithelium travel in a postero-anterior direction. The 'ascending ciliary current' of Stephenson (1913) does not exist.

4. The food boli of serpulids and sabellids rotate as they move down the gut. In *Salmacina incrustans* the rotation is imparted by cilia in the anterior part of the gut.

These observations were made in the Zoological Station of Naples. I wish to record my gratitude to the staff of the Station, to the British Association for the Advancement of Science for the use of its Table, and to the University of London for a grant towards travelling expenses.

## REFERENCES

BARRINGTON, E. J. W., 1937. Phil. Trans. B, **228,** 269.
CLAPARÈDE, E., 1873. Mém. Soc. Phys. Genève, **22,** 1.
EVENKAMP, H., 1931. Zool. Jb., Abt. 2, **53,** 405.
EWER, D. W., 1946. J. Mar. biol. Assoc. U.K., **26,** 426.
FAULKNER, G. H., 1930. J. Linn. Soc. (Zool.), **37,** 109.
GRAY, J., 1928. *Ciliary Movement.* Cambridge.
LINDROTH, A., 1938. Z. vergl. Physiol., **25,** 283.
—— 1941. Ibid., **28,** 485.
NICOL, E. A. T., 1930. Trans. Roy. Soc. Edinb., **56,** 537.
STEPHENSON, J., 1913. Ibid., **49,** 735.

# Variation in the Mouse Adrenal Cortex with Special Reference to the Zona Reticularis and to Brown Degeneration, together with a Discussion of the 'Cell Migration' Theory

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**With four Plates and six Text-figures**

## Contents

## 1. Introduction

THIS paper presents the results of a histological study of the adrenal gland of three strains of mice varying in age from 18 days to over 1 year. Two strains are normal (non-cancerous) and one cancerous (the RIII strain of Dobrovolskaia-Zavadskaia). The latter strain shows a peculiar spontaneous degeneration of the adrenal (named brown degeneration by Cramer and Horning, 1937, *a* and *b*). The origin of this degeneration, its variation with strain, sex, and age is given and its suggested relationship to cancer briefly discussed. Brown degeneration is compared with manifestations in the normal adrenal and this, together with an estimate of the amount of cell division in the adrenal cortex, is considered in the light it throws on the normal functioning of the gland with special reference to the 'cell migration' theory (that there is centripetal movement of cortical cells with replenishment from the periphery of the gland and destruction at the medullary border).

## 2. Material and Methods

The mice used are considered in two main groups, non-cancerous and cancerous, constituting in all 246 animals, 110 males and 136 females.

Group 1. The non-cancerous group is made up of two strains: (*a*) the Wistar albino strain (42 males and 52 females) inbred in this Department for 14 years with no case of spontaneous cancer, and (*b*) a 'piebald' strain (10 males and 19 females) inbred for 5 years with no case of spontaneous cancer.

Group 2. The cancerous group consists of two sub-strains developed from the RIII albino strain of Dobrovolskaia-Zavadskaia (Institut du Radium, Paris). The RIII strain shows a high incidence of mammary cancer in the female but none in the male. The two sub-strains are: (*a*) the CB sub-strain (23 males and 25 females) bred from a male and a female obtained in November 1938 from Dr. Bonser, Leeds University—her Z5/24 and 25; (*b*) the CBB sub-strain (35 males and 40 females) bred from 5 males and 2 females obtained in October 1939 from Dr. Bonser—her Z9/7–11 (males) and Z7/81 and 82 (females).

The mice were killed with chloroform. The chief fixatives used were Zenker, Helly, Susa, 5 per cent. formalin and Bouin, and the stains Ehrlich's haemotoxylin and eosin, Heidenhain's iron haemotoxylin and eosin, Mallory's connective tissue stain, Masson's acid fuchsin aniline blue stain. Sudan III was used for frozen sections after formalin fixation. The majority of the right adrenals were cut longitudinally at $5\mu$; the remainder and the left adrenals transversely at $5\mu$. Measurements were made on the right adrenal in all cases.

(i) Measurements (given in $\mu$) were made with a micrometer eye-piece. For estimates of the width of zones the mean is taken from three sections in each gland examined.

(ii) To estimate the size of the gland and the cortex/medulla relationship the 'paper replica' method was used. Only glands cut longitudinally were taken (the few cut transversely were excluded). The median section of each gland was projected ($\times 120$) on to paper (corrected to a standard weight) and the outline of the cortex and the medulla drawn. The paper replica of the whole section was weighed and that of the section minus the medulla. From this three figures are obtained for each gland.

1. A figure which represents the size of the median longitudinal section.

2. Similarly a figure for the size of the medulla.

3. The amount of cortex in the whole section expressed as a percentage.
These figures are taken to be representative of the whole gland. The method was extended to give the amount of brown degeneration present in advanced conditions. In these cases the outlines of the lobules of degeneration were also drawn and the amount of brown degeneration expressed as a percentage of the whole section.

(iii) The estimate of the amount of cell division in the cortex of a gland was made by examining five sections taken at random from the central bloc of the gland (i.e. sections towards the narrowed ends of the gland were excluded), and the number of mitotic figures in each section counted. The mean of the five sections was corrected for a standard area and the figure termed the 'mitotic index'. The standard area taken was the mean area of the female cortex in the median longitudinal section (as estimated from the

'paper replica' weight found in (ii) above). Where an X zone was present the mitoses were not included to give the 'mitotic index' but noted separately; they are not given in this paper as they confirm Whitehead (1933*c*).

Data were, where feasible, treated statistically by the methods of Fisher (1946).

### 3. Adrenal Size

Three factors are considered: the size of the adrenal, the size of the medulla, and the percentage of the cortex in the whole gland, the figures for which are obtained as given in Section 2 (ii) above. Adult males and adult parous females (i.e. which have had litters) are taken for comparison, that is, adult virgin females, in which the X zone (see Section 4) is an added complication, are excluded. The animals consist of (*a*) the non-cancerous group—33 males, age range 60 days to 375 days; 34 females, 119 to 403 days; (*b*) the cancerous group—35 males, 60 to 546 days; 36 females, 87 to 456 days. No statistically significant difference was found between mice of the same sex in the non-cancerous and the cancerous group and therefore the results for the two groups are given together and summarized in Table 1. The size of the adrenal and the size of the medulla in the male are expressed as a percentage of the female.

TABLE 1. *Non-cancerous and Cancerous Group Adult Males compared with the Adult Parous Females to show Relative Size of the Adrenal and of the Medulla and the Percentage of the Cortex in the Whole Gland*

(Figures obtained from the median longitudinal section of each gland. S.D.—Standard Deviation; S.E.—Standard Error)

| *All groups* | *No.* | Size of adrenal | | | Size of medulla | | | Per cent. cortex | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *S.D.* | *S.E.* | | *S.D.* | *S.E.* | | *S.D.* | *S.E.* |
| Males | 68 | 74·30 | 12·50 | 1·51 | 97·47 | 22·74 | 2·75 | 75·64 | 5·19 | 0·82 |
| Females | 70 | 100 | 24·90 | 2·97 | 100 | 27·12 | 3·24 | 82·09 | 3·54 | 0·42 |

From the Table three conclusions are drawn:

1. The gland is bigger in the female than in the male, the difference being about 25 per cent.

2. The medulla is about the same size in males and females. The females show a higher mean, but the difference is not significant.

3. The cortex, expressed as a percentage of the whole gland, is larger in the female than in the male, the difference being about 7 per cent. (This sexual dimorphism can be seen in Pl. I, figs. 1–4.)

The larger adrenal in the female is typical of a number of mammals (review, Parkes, 1945), and has been noted in the mouse by several workers. Only Carlson, Gustaffson, and Möller (1937) give a quantitative estimate. They considered 16 male and 17 female mice all 1 year old and found that the ratio

of the female adrenal weight to the male was as 100 to 47·6 (100; 49·7 corrected for body-weight), that the cortex in the female was 82·66 per cent. of the whole gland, the male 67·55 per cent., and that the medulla was about the same size in males and females. Their results bring out the same points as do mine, the differences in actual figures being due to the variation in method and also probably to the wider range of material in my data.

### 4. THE NORMAL ADRENAL

The description in this section is of the adrenal of the non-cancerous group of mice, but it applies equally to the cancerous group except when in the latter the picture is altered by the supervention of brown degeneration (Sections 5 and 6).

The outer connective tissue capsule, the zona glomerulosa, and the zona fasciculata will not be considered in detail. These layers correspond to the general mammalian pattern and have been described by other workers (Kolmer, 1918; Hett, 1926; Masui and Tamura, 1926; Howard-Miller, 1927; Deanesly, 1928; Whitehead, 1933b; Carlson *et al.*, 1937). Pl. 1, figs. 1–5, show the slight variation in general form in these zones although the inner zones differ in the different types of adrenal.

*The X Zone.* This zone, so designated by Howard-Miller (1927), has been the subject of much research; its function, if any, is still a matter of speculation (see Waring, 1942, for bibliography). My observations on its histological expression are confirmatory of other workers, but a brief review is necessary to obviate any confusion when the zona reticularis and brown degeneration are described. The X zone, the innermost cortical zone surrounding the medulla, is present in immature males and virgin females, and it is absent from mature males and females which have been pregnant. In an undegenerated condition the tissue has a distinctive character, characterized in sections stained with Ehrlich's haemotoxylin and eosin after Bouin by a smooth unbroken appearance, very eosinophil, densely packed and non-vacuolated cytoplasm, the nuclei spherical and of the cortical type. In the male the zone is obvious up to about 28 days, occupying, at the most, 15 per cent. of the cortex in mice of both groups. After this age the zone begins to collapse, the nuclei become pycnotic, and by 38 to 40 days the zone has completely disappeared. In the female the zone does not degenerate at maturity as in the male but increases in size until it forms a broad band around the medulla which may occupy as much as 48 per cent. of the cortex in mice of both groups. My data contain no virgins beyond 3 months, by which age the zone shows some sign of degeneration. It is clear from the literature, however, that the zone gradually disappears with age, usually by 'fatty degeneration' and vacuolization but also by collapse of the cells and pycnosis of the nuclei as in the male (Howard-Miller, 1927, 1939; Deanesly, 1928; Whitehead, 1933a). The X zone disappears during the first pregnancy by the twelfth day and often earlier (Tamura, 1926; Howard-Miller, 1927; Deanesly, 1928; Takewaki, 1936).

*The Zona Reticularis.* The condition of the innermost cortical zone after the disappearance of the X zone in the adult male and in the primi- or multi-parous female is not so clear. Confusion, which occasionally still persists (Kreyberg and Eker, 1939; Parkes, 1945, p. 204), has been caused largely because Masui and Tamura (1926) described the special zone, later termed the X zone, as the zona reticularis. Howard-Miller (1927) states that there is no recognizable zona reticularis in the male. Deanesly (1928) figures a zona reticularis in the male, and in the female, after the disappearance of the X zone, considers that a new cortical zone arises which may be transitory. Whitehead (1933, *a* and *b*) doubts the presence of a zona reticularis in the mouse adrenal. Carlson *et al.* (1937) describe a well-developed zone in females a year old, with it little developed or absent in coeval males.

*In the non-cancerous group parous female*, with an age range of 119 to 403 days, a zona reticularis is always present although variable in extent. This applies also to the CBB sub-strain females, age range 115 to 410 days, but not to those females of the CB sub-strain with an advanced condition of brown degeneration (Section 5). The zona reticularis begins where the regular arrangement of the zona fasciculata cells ceases (Pl. I, figs. 3 and 4); there is a transition from the cells of the inner region of the latter zone, which are more deeply eosinophil than those of the outer, to those of the zona reticularis. This zone is characterized by cells of the cortical type in which the cytoplasm is very eosinophil and the cells themselves in stages from slight shrinkage to complete collapse (Pl. II, figs. 6, 7, and 8). The nucleus rarely retains its spherical shape but is in some stage of pycnosis, and in a well-defined zone the nuclei are completely pycnotic. The zone is always vascular and sometimes hyperaemic (Pl. II, fig. 7). Fibrous strands run between groups of cells and are continuous with those of the medullary connective tissue capsule (Pl. II, figs. 6 and 8). The zone is very variable in width not only as between individuals but in the same gland. In some the zone is almost absent from parts of the gland, the zona fasciculata abutting on to the medullary capsule (Pl. II, fig. 9). In others it is as much as $150\mu$ in width, hyperaemic, and made up of completely collapsed cells and pycnotic nuclei staining jet-black with haemotoxylin. Where the gland in transverse section is elliptical, the reticular zone is widest and most prominent at the two narrowed ends of the gland (Text-fig. 1). The zone, in the majority of cases in the non-cancerous group, does not stain with Sudan III—it is 'lipoid' free (Pl. II, fig. 10). In some older mice small lipoid areas are seen in the zona reticularis (Pl. II, fig. 11), and these will be discussed in Section 6 below.

*In the mature male*, of the non-cancerous group after the disappearance of the X zone, a zona reticularis is largely absent (Pl. I, figs. 1 and 2) and the appearance of the innermost cortical zone is shown in Pl. III, fig. 12. The cells of the inner region of the zona fasciculata, which have eosinophil cytoplasm and spherical nuclei, abut directly on to the medullary capsule and there is no sign of degenerating cells. Even when present the zone is only slightly developed (Pl. III, fig. 13) and rarely completely encircles the medulla,

often showing, in transverse sections, the distribution but not the extent given in Text-fig. 1. Of the 37 males, of the non-cancerous group, ranging from 60 days to 375 days, 20 had no clearly recognizable zona reticularis. In 17 the zone was apparent though usually with the limited distribution noted above. There is no correlation of the presence of a zona reticularis with age. The zone in the male has the same characteristics as described for the female and it does not stain with Sudan III.
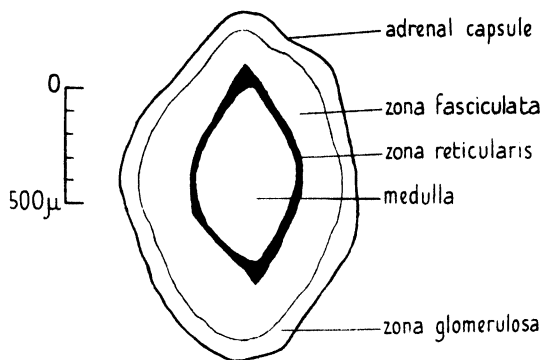


TEXT-FIG. 1. Diagram of a median transverse section of the adrenal of a normal parous female. The section shown is roughly elliptical and in these cases the zona reticularis is more prominent at the narrower ends.

In the immature male I cannot make out a zona reticularis although Waring (1935) figures one when the X zone is still present. In the virgin female up to 3 months (beyond which I have no data) the X zone is demarcated clearly from the zona fasciculata, and at this line of demarcation the fasciculata cells have a tendency to splay out and there are a few pycnotic nuclei visible, but this is not extensive enough to be termed a zone.

*The Medullary Connective Tissue Capsule.* This is a band of connective tissue lying between the cortex and the medulla (Pl. II, figs. 6, 8, 9, 12). According to Whitehead (1933c) and Carlson *et al.* (1937) it is more marked in the female than in the male, although Burrows (1945, p. 445) states that it is the male which has the well-developed capsule in comparison with the female.

In the male the medullary capsule appears at about 38 days concomitant with the disappearance of the X zone. It seems likely that the X zone contributes to its formation as Whitehead (1933a) and Waring (1935) suggest. From 50 to 60 days it is just discernible as thin strands of connective tissue. From this age onwards (up to 375 days in the non-cancerous group and to 546 days in the cancerous group—the latter showing the same extent and development of the capsule) it is an obvious but thin fibrous layer, of an average thickness of $3\mu$ in section, encircling the medulla as a neat band; there is no thickening with age. The thin capsule, the absence of a prominent

zona reticularis, and the smaller cortex makes the male adrenal recognizably different from the female in section (cf. Pl. I, figs. 1 and 2 with figs. 3 and 4).

In the parous female the medullary capsule is generally thick and obvious (Pl. II, figs. 6 and 8). It does, however, vary greatly in thickness in the same gland, from as much as $80\mu$ to $3\mu$. An average thickness is therefore difficult to arrive at, but for the 26 mice of the non-cancerous group, age range from 119 to 403 days, it is about $20\mu$. The capsule is irregular in appearance, pushing into the medulla and more peripherally being continuous with the reticulum of the zona reticularis. The capsule is frequently interrupted by the cortical-medullary blood-vessels.

### 5. The Origin and Formation of Brown Degeneration

In 1936 Cramer and Horning showed that the prolonged application of oestrin to mice produced a degeneration in the adrenal which, in 1937, they named brown degeneration and noted the spontaneous occurrence of a similar phenomenon in the RIII strain. Since then it has been observed, occurring spontaneously, in other—mainly cancerous—strains of mice (Dobrovolskaia-Zavadskaia, 1937; and Zephiroff, 1938; and Pezzini, 1939; Kreyberg and Eker, 1939; Blaisdell, Gardner, and Strong, 1941; Bonser, 1941; Kreyberg, 1941), and its appearance after oestrogens confirmed (Burrows, 1936; Lacassagne and Raynaud, 1937; Danner, 1938; Bonser and Robson, 1940). Earlier, although not named as such, Kolmer (1918) briefly mentions the same condition in mice of unknown age and origin and Hett (1926) describes its appearance in adrenals of mice after intermittent starvation. The physiological significance of brown degeneration is not known, but later work has usually described it in connexion with mammary cancer, and this will be discussed below (Section 6). Its site and mode of origin have been the subject of conflicting opinions. Burrows (1936) considers that it arises, in oestrin-treated adult male mice, from a re-established X zone or from cells in that location (1945); Dobrovolskaia-Zavadskaia (1937) that the degeneration is due to phagocytic action; and Lacassagne and Raynaud (1937) that it is the phagocytes themselves which have been mobilized and are degenerating. Kreyberg and Eker (1939) state that the origin of the degenerating cells is not always clear but that the main localization is in the connective tissue between cortex and medulla. Cramer and Horning (1939a) draw a distinction between its origin in oestrin-treated mice and those in which it arises spontaneously. In the former they consider that the degeneration arises in the zona reticularis and in the latter its chief site of origin is the medulla. Burrows (1945), however, states that he has never seen medullary cells involved in the degeneration.

*Brown Degeneration in Females of the CB sub-strain.* Brown degeneration is present to a greater or less extent in both cancerous and non-cancerous groups. It is, however, the CB females (which have been pregnant) from the age of 286 days onwards which show the most advanced condition. In this

the degenerative tissue consists of large syncytial masses forming a more or less continuous ring between the cortex and the medulla (Pl. I, fig. 5; Pl. III, figs. 14, 15, 16); some masses lie against the medullary capsule, some lie embedded within it (Pl. III, fig. 14), and others lie in the periphery of the medulla bounded to the outside by the capsule (Pl. III, fig. 15). In addition small irregular islets are found right in the medulla itself (Pl. III, fig. 16). As many as 60 well-developed lobules can be seen in sections of the glands showing the most advanced condition, and no zona reticularis, as defined for the normal adrenal (Section 4), is evident. The lobules themselves vary greatly in size from as much as $170\mu \times 170\mu \times 205\mu$, as measured in serial section, right down to small but clearly recognizable ones of $12\mu \times 6\mu \times 10\mu$. Each lobule is made up of finely vacuolated cytoplasm with occasional larger vacuoles, the cytoplasm being brownish in colour in unstained material and retains its brownish tinge after Ehrlich's haemotoxylin and eosin. It is this colour which gives the name to the degeneration. The lobules stain after Heidenhain's iron haemotoxylin and may be termed 'siderophil' (Pl. I, fig. 5); they are orange-red after Sudan III, i.e. 'lipoid' (Pl. III, fig. 16). Scattered throughout the lobule are numerous nuclei, as many as 30 being counted in a section. These nuclei are either completely pycnotic or are in some stage of pycnosis (Pl. III, figs. 14 and 15). Some, less degenerate than the rest, are clearly cortical in type; occasionally a cell outline may be observed about them (Pl. III, fig. 17).

Stages in the formation of brown degeneration have been found in CB females as early as 87 days. The earliest recognizable stages are in the cells of the inner region of the zona fasciculata lying two or three cells from the zona reticularis, this latter zone being discernible in all but the advanced condition of degeneration. The first sign is a small but distinct vacuole in such a cell (Pl. IV, fig. 18); further vacuolization occurs (Pl. IV, fig. 19); the cytoplasm becomes less eosinophil and finally does not take the stain at all; the nucleus shows pycnosis and the vacuolization spreads to include all the cell (Pl. IV, fig. 20). Such cells stain with Sudan III. Two such cells may be found together usually in the last row of the inner region of the zona fasciculata proper (Pl. IV, fig. 21). Coalescence of degenerating cells takes place and small lobules are formed with earlier stages of degeneration apparent nearby (Pl. IV, fig. 22). The nuclei are in various stages of pycnosis, the cytoplasm vacuolated with the characteristic brownish tinge now evident, but the original cortical cells are often obvious. Small lobules may be found bordering on to the zona reticularis (Pl. IV, fig. 22) or right within this zone, surrounded by the degenerated cells and blood spaces characteristic of the normal zone (Pl. IV, fig. 23). Fully formed but small lobules can be seen pressed together (Pl. IV, fig. 25) and by coalescence larger lobules are produced (Pl. IV, fig. 26). These formative stages are all found in the cortex itself, occurring right up to the medullary capsule. As the degeneration becomes more advanced, by further coalescing of lobules, masses are seen in the blood spaces which break the medullary capsule at intervals, and in more

advanced conditions lobules are sometimes found on the medullary side of the capsule (Pl. III, fig. 15). These lobules, although in the peripheral part of the medulla, are clearly made up of degenerated cortical cells. In the most advanced condition seen the medullary cells themselves degenerate, forming small irregular islets scattered infrequently throughout the medulla. The cytoplasm is vacuolated, brown in colour, and the nuclei degenerating (Pl. IV, fig. 26). The differentiating feature of the medullary islets is that the degeneration remains in areas delimited by the medullary reticular pattern without coalescence, and the rounded, compressed, lobules typical of the cortical degeneration are not formed.
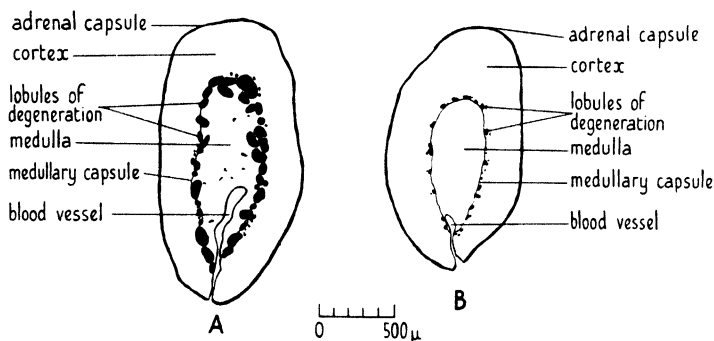


Text-fig. 2. Diagram of the median longitudinal section of the gland showing the most advanced condition of brown degeneration in (*a*) CB females; (*b*) CBB females.

*Brown Degeneration in Females of the CBB Sub-strain.* In females of this sub-strain of the RIII strain an advanced condition of brown degeneration is not seen. Text-fig. 2, *a* and *b* gives a comparison of the most advanced condition found in the CB and CBB females.

The lobules remain small, the largest seen was $45\mu \times 30\mu \times 50\mu$ measured in serial sections (as against $170\mu \times 170\mu \times 205\mu$ in the CB female), and the median section of the lobule showed 9 nuclei in various stages of pycnosis (as against 30 in the CB female). The stages in the formation of these lobules are the same as described above for the CB female. The very early stages are found in the zona fasciculata, and the lobules themselves are found scattered throughout the zona reticularis (Pl. IV, fig. 23, although of a CB female, shows the position and type of many of the lobules in the CBB female). The reticular zone is readily identifiable in this sub-strain and has the same characteristics and extent as given for the normal female mouse (Section 4). All the lobules are found on the cortical side of the medullary capsule; they may be found against the capsule or embedded within it, but in no case was the degeneration found within the medulla itself. Many of the fully formed lobules in the CBB sub-strain do not show the brown coloration and, remaining unstained after Ehrlich's haemotoxylin and eosin, show up with a grey appearance in the darkly stained zona reticularis.

*Brown Degeneration in the Males of the CB and CBB Sub-strains.* In the males the lobules are small and not numerous. Their development to this stage is identical with that given for the females. The lobules lie in the inner region of the zona fasciculata near to or against the medullary capsule or in the zona reticularis when this is present. In no case has the degeneration been seen in the medulla.

*Brown Degeneration in the Non-cancerous Group.* The degeneration is not found in the male, but it occurred in four older female mice (see Section 6). The condition was not advanced, a few small lobules being seen in the zona reticularis (Pl. II, fig. 11) with the early stages of formation apparent as described above for the cancerous group. There is no degeneration in the medullary capsule or in the medulla.

It seems clear that brown degeneration is primarily cortical in origin. It originates in the inner region of the zona fasciculata and lobules are formed from the degenerating cells. These lobules, which increase in size by coalescence, are found throughout the zona reticularis. Cells of this zone, which have degenerated in the manner typical of the normal mouse adrenal and which are evident in the adrenals of both groups except those CB females which show the most advanced condition of brown degeneration, may themselves become incorporated in the lobules. In later stages the masses of degenerating tissue are found meshed in the strands of the medullary capsule, although they do not arise there, and, freed from them, the lobules occur in the peripheral part of the medulla. Only in very advanced conditions are the cells of the medulla involved, and these only to a slight extent, forming small and easily recognizable islets scattered throughout the medulla. The X zone does not enter into the formation of brown degeneration as this zone has long since disappeared from the mice in which the condition has been described above, i.e. they are adult males and females which have been pregnant. The part played by the reticulo-endothelial system has not been elucidated as its components are displayed principally by vital staining methods (Capell, 1929; Jaffé, 1938; Polak, 1946). It is certain that the lobules are made up of actual cortical cells which have degenerated (and of medullary cells also in some cases) and not of phagocytes. I do not know, however, whether or not the lobules in advanced conditions are added to by members of the reticulo-endothelial system.

### 6. Brown Degeneration and its Relationship to Mammary Cancer

Cramer and Horning (1936, 1937, *a* and *b*, 1939, *a* and *b*, 1941) and Cramer (1940) maintain that there is a definite connexion between brown degeneration and mammary carcinoma. This is largely based on the fact that mice, after oestrogens, may produce mammary cancer and also show brown degeneration of the adrenal and that the RIII strain of mice, with a high incidence of mammary cancer, have an advanced condition of brown degeneration from the age of 6 months onwards. They point out, however, that

the degeneration is not in itself the cause of mammary cancer since in the high cancer strains it develops in practically every mouse, male or female, whether the mammae of these animals are cancerous or not; nor is the development of cancer absolutely dependent on its presence in every case, since in the low-cancer Bagg albino strain and mixed strains mammary cancer may develop with the adrenal intact. They suggest that the aetiology of mammary cancer may be found in an endocrine imbalance, and the spontaneous development of brown degeneration in the adrenal medulla is one of the ways in which this may be brought about. Dobrovolskaia-Zavadskaia and her co-workers (1937, 1938, 1939) conclude that there is some not yet understood connexion between the two phenomena, and the tendency to brown degeneration may be *en rapport* with some endocrine disturbance. Kreyberg and Eker (1939) agree with this general view. Bonser (1941), summarizing the position, expresses doubt as to the existence of any aetiological relationship between cancer and degeneration of the adrenal. Blaisdell *et al.* (1941) reach the same conclusion and suggest, in addition, that, as brown degeneration is produced by the oestrogens and that these too cause hypofunction of the pituitary, brown degeneration may be consequent upon the underfunctioning of the pituitary and especially upon an inadequate amount of adrenocorticotrophin.

In order to give an estimate of the amount of degeneration present in an adrenal a grading scheme was adopted. It is similar to that of Blaisdell *et al.* (1941) except that the higher grades are estimated quantitatively by the method given in Section 2. The grades are as follows:

0. No brown degeneration.
1. Early stages visible and a few small lobules (i.e. very little).
2. Obvious lobules scattered sparsely throughout the zona reticularis or, in the case of some males where the zone is absent, in the inner region of the zona fasciculata (i.e. little).
3. The degeneration consists of fully formed lobules with numerous early stages and occupies 1–3 per cent. of the median longitudinal section.
4. The degeneration occupies 3–6 per cent.
5. The degeneration occupies 6–9 per cent.
6. The degeneration occupies 9–12 per cent.

The data for males of the CB and CBB sub-strains are given in Text-fig. 3, and for females in Text-fig. 4, where in each case the grade of degeneration is plotted against age (from the age at which it first appears in the sub-strains). Those females which had a mammary carcinoma at death (designated by suffix C in Text-fig. 4) are also shown.

*CB and CBB males* (Text-fig. 3). The age of onset of brown degeneration is about 140 days; only a slight amount of degeneration is shown, the grade never being higher than 2; from 143 to 286 days CB males (with 17 out of 19)

are more inclined to have brown degeneration than coeval CBB males (8 out of 16); of the 5 mice above this age 1 is Grade 0 (420 days), 3 are Grade 1 (365, 418, 495 days), and 1 Grade 2 (546 days).

*CB and CBB Females* (Text-fig. 4). The degeneration is generally established at 142 days in the CB sub-strain with one early example at 87 days, and at 153 days in the CBB. CB females (with 8 out of 22 Grade 3 and above) show a significant difference from the CBB females (28 mice with the grade in no case higher than 2) in the amount of brown degeneration. A fairly advanced condition first appears in the CB sub-strain at 186 days and an advanced condition is well established at 286 days and above with one exception (Grade 2) at 414 days. Both sub-strains maintain a high incidence of
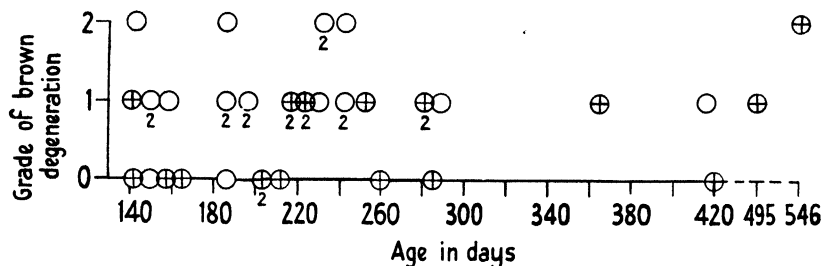


TEXT-FIG. 3. The grade of brown degeneration in the right adrenal of CB and CBB sub-strain males plotted against age (from the age of the first appearance of the degeneration). ○ CB males. ⊕ CBB males. Numerical suffix: number of individuals at that point.

cancer formation—8 CB females with mammary cancer out of 9 from 246 to 456 days, and 9 CBB females with mammary cancer out of 19 from 258 to 410 days—but the data are not complete enough to compare incidences. There is no correlation between brown degeneration and the appearance of a mammary cancer. Also one mouse (316 days) had a mammary cancer and no brown degeneration. Brown degeneration is generally well established before the usual age of onset of cancer formation.

*Brown Degeneration in the Non-cancerous Group*. The males of this group did not show the degeneration, and it was rare in the females: of the 8 females from 322 days to 403 days, 4 had brown degeneration. These were 1 at 322 days (Grade 1), 1 at 359 days (Grade 2), and 2 at 403 days (Grade 1).

The interest in this section lies in the fact that the new data show that even in the RIII strain a sub-strain has been developed which does not give an advanced condition of brown degeneration yet maintains a high incidence of cancer formation. It appears probable that the existence of some high cancer strains with advanced condition of brown degeneration is coincidental, and that there is no correlation between the two phenomena. In strains of which it is not characteristic it may appear in older females, as shown by those of the non-cancerous group, perhaps as a phenomenon of ageing.

## 7. Cell Division in the Adrenal Cortex

The method for obtaining the amount of cell division per standard area for each gland—termed the mitotic index—is given in Section 2. The data for the males of the non-cancerous group, the CB sub-strain, and the CBB sub-strain are given in Text-fig. 5, and for the females in Text-fig. 6. In each case the mitotic index for each individual is plotted against age.

*Males* (Text-fig. 5). At the youngest age taken, 18 days, the amount of cell division is great (a mean mitotic index of 45 for 5 mice). After this age the
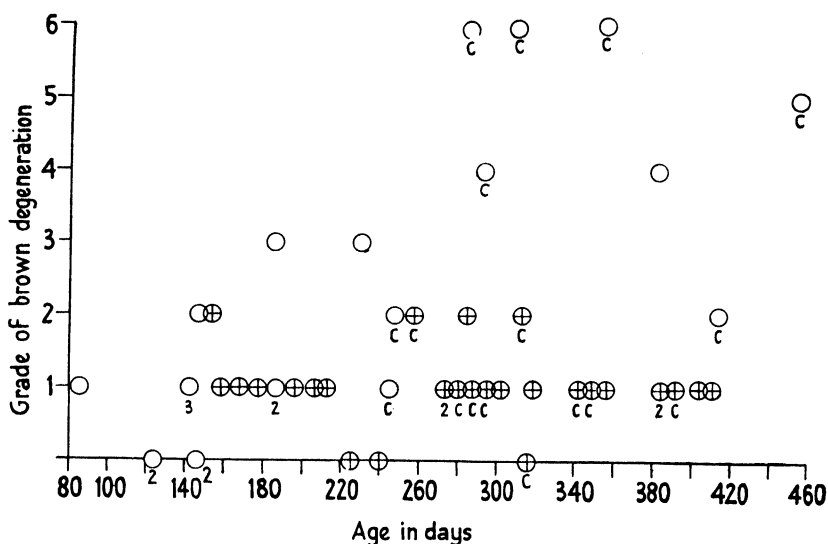


TEXT-FIG. 4. The grade of brown degeneration in the right adrenal of CB and CBB sub-strain parous females plotted against age (from the age of the first appearance of the degeneration) ○ CB females; ⊕ CBB females; suffix C—mammary cancer present at death. Numerical suffix: the number of individuals at that point.

rate falls off rapidly but remains relatively high up to about 60 to 70 days. From 70 to 286 days mitoses are rare and may be regarded as forming part of a Poisson population with a mean number of 1·66 for the 64 mice in the group. For statistical treatment the data were converted to a more normal population by using the conversion formula $\sqrt{(n+\frac{1}{2})}$ where $n$ is the number of mitoses. The variance was found to be about the same over this group. It was found that there is no significant difference between the three sets of males, non-cancerous, CB, and CBB, and that there is no correlation of the amount of cell division with age. Of the 7 mice of 1 year and over, 6 show no cell division and 1 (CBB at 365 days) an index of 1·01. It is probable that there is a falling off in the rate of cell division in the older male mice.

*Females* (Text-fig. 6). At the younger ages the amount of cell division is great. From 60 to 115 days there is a wide variance from a mitotic index of 1 to 11, and these were not analysed statistically. From 115 to 456 days all the mice in the three sets—non-cancerous, CB, and CBB—have been pregnant (this excludes one primiparous mouse, a CB aged 87 days). These females are considered in two groups, 115 to 359 days and 380 to 456 days, there being no observations between 359 and 380 days. The data are regarded
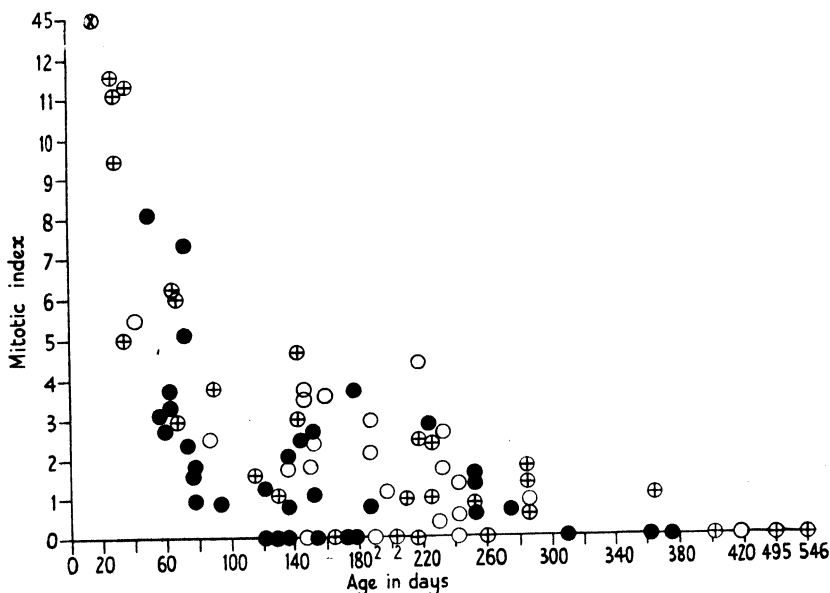


TEXT-FIG. 5. The mitotic index of males plotted against age. ○ CB sub-strain; ⊕ CBB sub-strain; ● non-cancerous group; ⊗ mean of 5 readings (CB, 1; CBB, 2; N–c, 2). Numerical suffix gives the number of individuals at that point.

as being drawn from a Poisson population. The 115–359-days group, of 59 mice, has a mean number of 1·74. Using the conversion formula as given for the males, the group was found to be just about homogeneous although the variation is wide in a few cases. There is no significant difference between the three sets of females—non-cancerous, CB, and CBB—and there is no correlation of mitotic index with age. The second group, 380–456 days consisting of 12 mice, has a mean number of 0·71. Using the conversion formula it was found that this lower mitotic index is significantly different from the 115–359-days group.

Comparing the adult males of all types from 70 to 286 days of age with the parous females from 115 to 359 days it was found that there is no significant difference between the mitotic indices (i.e. 64 males with a mean of 1·66 and 59 females with a mean of 1·74).

There are few mitotic counts for the adrenal of the Mammalia as a whole (see Blumenthal, 1940, 1945) and I am aware of only that of Whitehead's (1933c) for the mouse adrenal. He examined three sections from each gland of 107 right and left adrenals from 57 males aged 15 to 336 days and 115 from 61 females aged 15 to 338 days. Up to 60 days his data show the same pattern as mine in both males and females. For male mice aged 85–336 days (45 mice, 87 glands) his mean figure is 1·00 and this is the same order as my mean of 1·66 for 64 mice aged 70–286 days, especially in view of the fact that
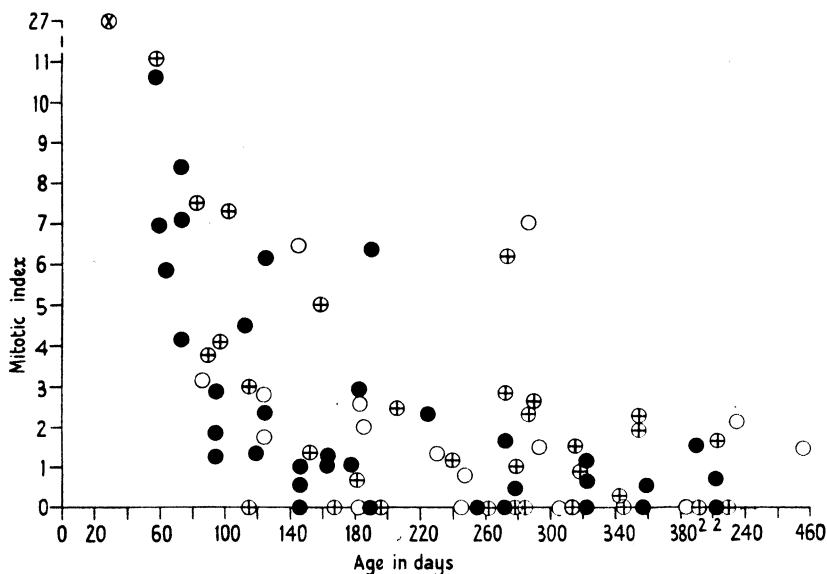


TEXT-FIG. 6. The mitotic index of females plotted against age. ○ CB sub-strain; ⊕ CBB sub-strain; ● non-cancerous group; ⊗ mean of 3 readings (CB, 1; CBB, 1; N-c, 1). Numerical suffix gives the number of individuals at that point.

my figures are corrected for a standard area—that of the female cortex—and the male adrenal is smaller than the female (Section 1). For the females aged 85–388 days (46 mice, 91 glands), his mean of 0·42 is much lower than mine of 1·74 for 59 mice aged 115 to 359 days. The correction to a standard area for my data would not account for the difference. There are undoubtedly many unknown factors which influence cortical cell division; it is not clear if the females he took were virgins or had been pregnant; further, Baxter (1946) notes differences between his own data and those of Nathanson and Brues (1941) for the rat, and Blumenthal (1940) between his for the guinea-pig and those of Schmidt and Schmidt (1938) for which methods of sampling by no means account. Whitehead's data do show activity up to the oldest age he examined—for the 8 glands of his male 336-day group the mean is 2·62 and for 8 glands of coeval females the mean is 1·12, and my data show cell division

up to a year in the male and 456 days in the female. There is an indication in my data that the mitotic activity is less over 1 year in both males and females although mitoses are still found in the latter. The function of cell division in the cortex will be considered in the Discussion (Section 8), but it is worth noting here the absence of a clear difference in the mitotic indices of the non-cancerous and cancerous groups, especially as regards the females. For if a causative factor of brown degeneration is the liberation of excess oestrin (Cramer, 1940) it might be expected the CB females would show a significantly high rate of cell division as oestrogen treatment causes a marked increase in the number of mitoses in the rat adrenal cortex (Ellison and Burch, 1936). This might not apply if the action, in cases of the spontaneous occurrence of the degeneration, is via the pituitary (Blaisdell *et al.*, 1941).

*The Site of Cell Division.* There is no difference between the site of cell division in the non-cancerous and cancerous groups or between males and females. In immature animals mitoses are found throughout the cortex both in the 'permanent' cortex and the X zone. In adult animals the majority of mitoses are found in the inner part of the zona glomerulosa, about 3 to 6 cells in from the outer connective tissue capsule (Pl. IV, fig. 27). The zona glomerulosa, in this region, begins to show the transition to the type of cells found in the outer part of the zona fasciculata. Mitoses are also found, however, anywhere in the zona glomerulosa from this position right up to the outer capsule itself (Pl. IV, fig. 28). Mitoses are occasionally found in the outer part of the zona fasciculata. I have not observed them in the inner region of the zona fasciculata or the zona reticularis. Mitoses are rare in the outer connective tissue capsule and I have not observed one there above 100 days in any group.

Most authors agree that the site of the majority of the cell division in the cortex of the adult mammal is the transition layer consisting of the inner part of the zona glomerulosa and the outer part of the zona fasciculata (Hoerr, 1931; Whitehead, 1933; Bennett, 1940; Blumenthal, 1940; Gruenwald and Konikov, 1944). It seems, from my observations, that the whole of the zona glomerulosa is capable of cell division. I have seen no evidence, in the mouse, for the mechanisms of cell replacement from the adrenal capsule as described for other mammals by Zwemer, Wotton, and Norkus (1938), Salmon and Zwemer (1941), Wotton and Zwemer (1943), Gruenwald and Konikov (1944). These authors do not specifically include the mouse and as the adrenal capsule in this animal is a clear-cut, narrow zone (Pl. IV, fig. 27), with no histological evidence for the transformation of capsular cells to glomerular ones as they figure in other mammals, it is doubtful if their theories would apply to the mouse; Gruenwald and Konikov do in fact exclude mammals with a narrow distinct outer capsule. The germinative role of the capsule seems to be confirmed by the regeneration experiments from enucleated glands (Ingle and Higgins, 1938; Turner, 1939). But, as Ingle and Higgins themselves point out, it is not certain that all glomerular tissue is removed by enucleation and as, in the mouse certainly and in other mammals possibly, cell division of

glomerular cells may take place right against the outer connective tissue capsule, the evidence is not conclusive.

## 8. Discussion

The 'Cell Migration' or 'Escalator' theory, by which it is considered that the cortical cells are constantly degenerating at the medullary border and as constantly being renewed by multiplication at the periphery and by migration inwards, with cytomorphosis, of the cells so formed, is widely accepted (review up to 1931 by Hoerr; Ivanov, 1932; Zwemer, 1936; Waring and Scott, 1937; Zwemer, Wotton, and Norkus, 1938; Bennett, 1940; Salmon and Zwemer, 1941; Wotton and Zwemer, 1943; Gruenwald and Konikov, 1944; Baxter, 1946; Gruenwald, 1946). The histological appearance of the adrenal of the normal parous female mouse fulfils the two conditions upon which this theory is largely based—that there is cell division at the periphery of the gland, and, at the medullary border, there is a zone of senescence, the zona reticularis. Experimental evidence for the theory is lacking (Calmo and Foster, 1943). The histological facts in regard to the normal adult male adrenal are not so convincing. There is certainly cell division at the periphery of the gland, equal per standard area to that in the female, for a large proportion of adult life. The zona reticularis, however, is at best little developed and often absent. The volume of the cortex is smaller in the male and the cell pressure at the medullary border would not be so great and therefore the zona reticularis would not be expected to be so extensive as in the female. But it is its frequent absence in the male which is surprising if, as the theory requires, there is constant cell destruction in the innermost cortical layer.

If, then, cortical cell division is not concerned, partly or solely, with cell replenishment, it could be evidence of the growth of the gland. Whitehead (1943), discussing the guinea-pig, considered that cortical mitotic activity merely meant that growth was taking place and that the theory of cell migration is not tenable. If this is so it would follow that the mouse adrenal is increasing in size throughout most of its adult life, as my data include mitoses in the male up to 365 days and in the female up to 456 days, Whitehead (1933c) shows cell division in the mouse up to 10 months, and, as regards the guinea-pig, Blumenthal (1940, 1945) finds mitoses in animals over 2 years of age. Data to show that the mouse adrenal increases in size throughout the major portion of its life are not available. For the male rat, however, Korenchevsky (1942), while pointing out the relative hypoplasia of organs with age, states that the majority of organs (including the adrenal) continue to grow in the adult, but that towards the middle or end of the period examined (21 to 500 days) the increase in actual weight becomes very slow or may even be stationary. So that the possibility that mitoses in the adult adrenal cortex are concerned with growth is not completely excluded.

This naturally leads to an examination of the alternative to the cell migration theory. By this alternative theory, which I will call for convenience the 'zonal' theory, the zona glomerulosa, the zona fasciculata, and the zona

reticularis are regarded as discrete layers both in structure and function. The functional separation of the first two zones is based on the results following hypophysectomy and ascribes the secretion of the carbohydrate metabolism regulating hormone to the zona fasciculata—which in the cell migration theory is regarded as the site of all cortical hormone production (e.g. Bennett, 1940) —and of the 'salt' regulating hormone to the zona glomerulosa (Swann, 1940; Sarason, 1943; Deane and Greep, 1946). The zona reticularis is considered to be associated with reproduction (review by Vaccarezza, 1946; Blackmann, 1946). There is considered to be no centripetal replenishment of these distinct zones from a germinative layer, but there may be local degeneration and regeneration in each zone (Tonutti, 1941, 1942, *a* and *b*; Vaccarezza, 1946). Deane and Greep consider that the mitoses observed in the inner margin of the zona glomerulosa may only be the regenerative region of the zona fasciculata and, in contrast, the cells of the zona glomerulosa may die locally and be replaced from the capsule. The zonal theory would find support, histologically, therefore, in evidence of cell division throughout the cortex and in the frequent presence of degenerating cells in zones besides the reticular, but such evidence is not forthcoming from the mouse adrenal.

The spontaneous degeneration of the adrenal of the RIII mice is of significance in the consideration of these two theories. For it is difficult to conceive of the advanced condition of brown degeneration, as seen in the CB female, arising except by the inward movement of cortical cells. It has been shown to be a purely cortical phenomenon in its initial stages. Its origin is in the cells of the inner region of the zona fasciculata. It appears as lobules in the zona reticularis which increase in size by coalescence; then in more advanced conditions the lobules are emeshed in the strands of the medullary capsule and freed from them in the medulla itself, still clearly made up of degenerated cortical cells. These facts give a very telling histological picture in favour of centripetal movement of cortical cells.

On the whole the balance of the histological evidence lies in favour of the classical cell-migration theory. The condition of the male mouse adrenal, in both the cancerous and non-cancerous groups, is, however, a challenge which is only partly met by the fact that the volume of the cortex is smaller than in the female. The resolution of the problem must await more experimental evidence.

A question remains in regard to brown degeneration. The factor causing this type of degeneration has not been elucidated, but if the cell-migration theory is accepted it is only another mechanism by which, in any case, the cells of the adrenal cortex degenerate as they reach the innermost cortical layer. Why, however, should there be accumulation of this degenerated material?—and this is a pertinent question, whether the zonal or the cell migration theory be the correct one. In the normal adrenal it is supposed that the dead cells of the zona reticularis are removed—as Hoerr (in Maximov and Bloom, 1946, p. 322) says: 'The cells of the cortex appear to be degenerating and dying continually in the zona reticularis. The debris is removed

mainly by the macrophages which are always present.' The reticulo-endo-thelial system is the obvious agent by which brown degeneration should be removed and its non-removal may therefore indicate that this system is not so efficient in the CB female, and, to a less extent, in the cancerous group as a whole. It is possible, further, that, as brown degeneration appears in older non-cancerous mice, loss of efficiency is normally a function of age. This is only a tentative hypothesis, but it is interesting to note that Jones (1947), working in this Department, found that haemosiderin accumulation in the uterus was removed by macrophages much less quickly in the RIII strain than in normal mice.

I am very grateful to Mrs. R. C. Bisbee for help and criticism, and to Mr. R. Plackett for aid with statistical methods.

### 9. Summary

1. The adrenals of two groups of mice, ranging in age from 18 days to over 1 year, were examined. One group is non-cancerous (52 males and 71 females); the other the RIII cancerous strain from which two sub-strains have been developed; (i) the CB sub-strain (23 males and 25 females); (ii) the CBB sub-strain (35 males and 40 females).

2. In the adults of both groups the parous female adrenal is bigger than the male by about 25 per cent.; the medulla is about the same size in males and females; in the female the cortex is 82·09 per cent. of the whole gland, in the male 75·64 per cent.

3. In the parous non-cancerous female, the zona reticularis is always present; in the adult male it is little developed and often absent.

4. Brown degeneration, found in the adrenal of the cancerous group, arises in the cells of the inner zona fasciculata; the subsequent formation of the typical lobules is traced. The medulla is involved in advanced conditions.

5. CB females show an advanced condition of brown degeneration, CBB females do not. Both maintain a high incidence of mammary carcinoma. Four older females of the non-cancerous group have a slight amount of brown degeneration. Cancerous males have little degeneration and non-cancerous males none.

6. The mitotic count per standard area in the cortex is given. There is no significant difference between the non-cancerous and cancerous groups or between adult males and parous females under 1 year of age. Over 1 year there is an indication that the amount of cell division falls off, although in the female mitoses are still found up to 456 days. Mitoses occur all through the zona glomerulosa with the majority towards the inner border.

7. The histological evidence is, on the whole, in favour of the 'cell-migration' theory (the centripetal movement of cortical cells with replenishment from the periphery and destruction at the medullary border). It is suggested that brown degeneration is a variation of the way in which cortical cells .

normally degenerate; but the causative factor is not known; it has no direct correlation with mammary cancer, and its occurrence as characteristic of certain cancerous strains may only be coincidental.

## 10. REFERENCES

BAXTER, J. S., 1946. J. Anat., Lond., **180**, 139.
BENNETT, H. S., 1940. Amer. J. Anat., **67**, 151.
BLACKMANN, S. S., 1946. Bull. Johns Hopkins Hosp., **78**, 180.
BLAISDELL, J. S., GARDNER, W. U., and STRONG, L., 1941. Cancer Res., **1**, 283.
BLUMENTHAL, H. T., 1940. Endocrinology, **27**, 477.
—— 1945. Arch. Pat., **40, 264**.
BONSER, G. M., 1941. Proc. 7th Int. Cong. Genet. (1939), 80.
—— and ROBSON, J. M., 1940. J. Path. and Bact., **51,** 9.
BURROWS, H., 1936. J. Path. and Bact., **43,** 121.
—— 1945. *Biological Actions of Sex Hormones.* Cambridge U.P.
CALMO, I., and FOSTER, C. L., 1943. Nature, Lond., **152,** 536.
CAPELL, D. F., 1929. J. Path. and Bact., **32,** 595.
CARLSON, H., GUSTAFFSON, B., and MÖLLER, K., 1937. Upsala Läk. Forhandl., **1**, 49.
CRAMER, W., 1940. Amer. J. Cancer, **38,** 463.
—— and HORNING, E. S., 1936. Lancet, Lond., **230, 237**.
—— —— 1937a. Nature, Lond., **139,** 196.
—— —— 1937b. J. Path. and Bact., **44,** 633.
—— —— 1939a. Amer. J. Cancer, **37,** 343.
—— —— 1939b. Lancet, Lond., **236,** 192.
—— —— 1941. Proc. 7th Int. Cong. Genet. (1939), 91.
DANNER, M., 1938. Klin. Wchns., **17,** 658.
DEANE, H. W., and GREEP, R. O., 1946. Amer. J. Anat., **79,** 117.
DEANESLY, R., 1928. Proc. Roy. Soc. B, **103,** 523.
DOBROVOLSKAIA-ZAVADSKAIA, N., 1937. C.R. Soc. Biol., **125,** 877.
—— and ZEPHIROFF, P., 1938. C.R. Soc. Biol., **128,** 971.
—— and PEZZINI, Z. M., 1939. C.R. Soc. Biol., **131,** 240.
ELLISON, E. T., and BURCH, J. C., 1936. Endocrinology, **20,** 746.
FISHER, R. A., 1946. *Statistical Methods for Research Workers*, 10th ed.  London (Oliver and Boyd).
GRUENWALD, P., 1946. Anat. Rec., **95,** 391.
—— and KONIKOV, W. M., 1944. Anat. Rec., **89,** 1.
HETT, J., 1926. Z. f. mik. anat. Forsch., **7,** 403.
HOERR, N., 1931. Amer. J. Anat., **40,** 251.
HOWARD-MILLER, E., 1927. Amer. J. Anat., **40,** 25.
—— 1939. Amer. J. Anat., **65,** 105.
INGLE, D. J., and HIGGINS, G. M., 1938. Endocrinology, **22,** 458.
IVANOV, M. J., 1932. Arch. Russ. d'anat. d'hist. d'embryol., **11,** 219.
JAFFÉ, R. H., 1938. *Handbook of Hematology*, vol. ii (Chap. 15), ed. H. Downey.  London (Hamish Hamilton).
JONES, V. E., 1947. Quart. J. micr. Sci. (in the press).
KOLMER, W., 1918. Arch. mikr. Anat., **91,** 51.
KORENCHEVSKY, V., 1942. J. Path. and Bact., **54,** 13.
KREYBERG, L., 1941. Proc. 7th Int. Cong. Genet. (1939), 178.
—— and EKER, R., 1939. Avhand. ut. av det Nor. Viden. Akad., Oslo I. Mat. Nat., **3,** 3.
LACASSAGNE, A., and RAYNAUD, A., 1937. C.R. Soc. Biol., **124,** 1183.
MASUI, K., and TAMURA, Y., 1926. J. Coll. Agric. Imp. Univ. Tokyo, **7,** 353.
MAXIMOV, A. A., and BLOOM, W., 1946. *Text-book of Histology*, 4th ed.  Phil. and London (W. B. Saunders).
NATHANSON, I. T., and BRUES, A. M., 1941. Endocrinology, **29, 397**.
PARKES, A. S., 1945. Phys. Rev., **25,** 203.
POLAK, M., 1946. Rev. Asoc. Med. Argent., **60,** 9.
SALMON, T. N., and ZWEMER, R. L., 1941. Anat. Rec., **80,** 421.

SARASON, E. L., 1943. Arch. Path., **35**, 373.
SCHMIDT, I. G., and SCHMIDT, L. H., 1938. Endocrinology, **23**, 559.
SWANN, H. G., 1940. Phys. Rev., **20**, 493.
TAKEWAKI, K., 1936. J. Facult. Sci., Tokyo, **4**, 277.
TAMURA, Y., 1926. J. exp. Biol., **4**, 81.
TONUTTI, E., 1941.[1] Z. f. mik. anat. Forsch., **50**, 283.
—— 1942a.[1] Z. f. mik. anat. Forsch., **51**, 346.
—— 1942b.[1] Z. f. mik. anat. Forsch., **52**, 32.
TURNER, C. D., 1939. Anat. Rec., **73**, 145.
VACCAREZZA, A. J., 1946. Rev. Asoc. Med. Argent., **60**, 9.
WARING, H., 1935. Quart. J. micr. Sci., **79**, 329.
—— 1942. J. Endocrinol., **3**, 123.
—— and SCOTT, E., 1937. J. Anat., Lond., **71**, 299.
WHITEHEAD, R., 1933a. J. Anat., Lond., **67**, 387.
—— 1933b. J. Anat., Lond., **67**, 393.
—— 1933c. J. Anat., Lond., **67**, 399.
—— 1943. J. Path. and Bact., **55**, 392.
WOTTON, R. M., and ZWEMER, R. L., 1943. Anat. Rec., **86**, 409.
ZWEMER, R. L., 1936. Amer. J. Path., **12**, 107.
—— WOTTON, R. M., and NORKUS, M. G., 1938. Anat. Rec., **72**, 249.

[1] Quoted from Gruenwald (1946).

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## EXPLANATION OF PLATES

### PLATE I

Fig. 1. A median longitudinal section of the right adrenal of an adult non-cancerous group male, aged 178 days. No zona reticularis evident. Bouin, Heidenhain's haem., Masson. × 40.

Fig. 2. A transverse section of the left adrenal of an adult cancerous group male, aged 186 days. No zona reticularis evident, the darker staining tissue around the medulla is the inner region of the zona fasciculata. This example has no brown degeneration and therefore is representative also of the adult non-cancerous male. Bouin, Ehrlich's haem. and eosin. × 40.

Fig. 3. A median longitudinal section of the right adrenal of a parous non-cancerous group female, aged 272 days. The deeply staining, irregular zona reticularis surrounding the medulla can be seen. Compare this gland with that of the male in Fig. 1. Bouin, Ehrlich's haem. and eosin. × 40.

Fig. 4. A transverse section of the left adrenal of a parous cancerous female, aged 146 days. The columns of the zona fasciculata cells are visible; they merge into the deeply staining zona reticularis which is clear but not extensive. This example has no brown degeneration and is therefore representative also of many non-cancerous group females. Bouin, Heidenhain's haem., and Masson. × 40.

Fig. 5. A longitudinal section (not quite median) of the right adrenal of a cancerous group parous female, CB sub-strain, aged 286 days, showing an advanced condition of brown degeneration (Grade 6). The lobules can be seen at the cortex-medulla border. No zona reticularis, as shown in Fig. 3, can be seen. Bouin, Heidenhain's haem., and Masson. × 40.

### PLATE II

Fig. 6. A portion of the adrenal of a non-cancerous parous female, aged 272 days, shown in Fig. 3. The cells of the inner region of the zona fasciculata merge into the irregular zona reticularis. The medullary capsule can be seen, and, below, the medulla. × 350.

Fig. 7. An extensive and vascular zona reticularis from a CBB parous female, aged 240 days. The gland shows no brown degeneration and the zona reticularis is characteristic also of some non-cancerous females. Bouin, Ehrlich's haem., and eosin. × 350.

Fig. 8. A part of the zona reticularis of a non-cancerous female, aged 403 days; it is a mass of senescent cells; the medullary capsule is very prominent. Bouin, Ehrlich's haem. and eosin, Mallory. × 350.

Fig. 9. Part of an adrenal of a non-cancerous parous female, aged 177 days, which shows no zona reticularis; the cells of the zona fasciculata abut on to the medullary capsule. Bouin, Ehrlich's haem., and Masson.   $\times 350$.

Fig. 10. A sector of the adrenal of a non-cancerous group parous female, aged 163 days, showing the 'lipoid' free zone between the zona fasciculata and the medulla. Five per cent. formalin. Sudan III.   $\times 40$.

Fig. 11. A sector of the adrenal of a non-cancerous group parous female, aged 359 days, showing small lobules of 'lipoid' material (small brown degeneration lobules) in the zona reticularis. Five per cent. formalin. Sudan III.   $\times 40$.

## PLATE III

Fig. 12. The innermost cortical layer of the adrenal shown in Fig. 1. No zona reticularis, the fuchsinophil cells of the inner zona fasciculata abut on to the medullary capsule which is thin.   $\times 350$.

Fig. 13. The zona reticularis of a non-cancerous group male, aged 152 days. It has the characteristics of the normal female zona reticularis but it is not so extensive. Bouin, Ehrlich's haem. and eosin.   $\times 350$.

Fig. 14. Part of the gland shown in Fig. 5. The top of the photograph shows cells of the inner zona fasciculata with stages in the formation of brown degeneration; lower, the vacuolated lobules with pycnotic nuclei typical of brown degeneration. Part of the medullary capsule is to be seen at the bottom of the photograph and then the medulla itself.   $\times 350$.

Fig. 15. Part of the right adrenal of a CB parous female, aged 310 days. Brown degeneration lobules are at the periphery of the medulla, bounded, on the cortical side, by the medullary capsule. The latter is interrupted centrally. The lobules consist of degenerated cortical cells. Bouin, Heidenhain's haem., Masson.   $\times 350$.

Fig. 16. A sector of the left adrenal of a CB parous female, aged 356 days. The lobules of brown degeneration are to be seen between the cortex and the medulla. Five per cent. formalin. Sudan III.   $\times 40$.

Fig. 17. A small portion of a large lobule of brown degeneration chosen to show the cortical nature of some of the nuclei which have not completely degenerated and the remains of the cell wall around one of them (bottom right). Bouin, Ehrlich's haem. and eosin. $\times 700$.

## PLATE IV (all figures $\times 700$)

Figs. 18–25. Stages in the formation of a lobule of brown degeneration. (From Bouin, Ehrlich's or Heidenhain's haem. and eosin preps.)

Fig. 18. A cell of the inner region of the zona fasciculata showing vacuolization.

Fig. 19. A similar cell with the vacuolization increased.

Fig. 20. A cell from the same region, the cytoplasm completely vacuolated, the nucleus showing pycnosis.

Fig. 21. Two cells of the inner region of the zona fasciculata which have merged. The cell walls are barely discernible, the cytoplasm vacuolated, the nuclei showing pycnosis.

Fig. 22. A small lobule of brown degeneration formed by the coalescence of five cells. The nuclei have not completely degenerated and are of the cortical type. The lobule is situated in the last row of zona fasciculata cells bordering on to the zona reticularis.

Fig. 23. A small lobule of brown degeneration lying against the medullary capsule and surrounded by the blood spaces and collapsed cells typical of a normal zona reticularis.

Fig. 24. Two small lobules of brown degeneration coalescing to form a larger.

Fig. 25. A larger lobule of brown degeneration lying against the medullary capsule. The degenerating nuclei are of the cortical type, the cytoplasm brown and vacuolated.

Fig. 26. Medullary cells showing brown degeneration. The reticulum of the medulla can be made out. Note the larger size of the medullary nuclei, compared with the cortical in Figs. 18–25.

Fig. 27. A usual position for a mitotic figure in the zona glomerulosa. Also note the narrow, clearly defined, adrenal capsule. Bouin, Ehrlich's haem. and eosin.

Fig. 28. A cell of the zona glomerulosa, dividing. It is against the adrenal capsule. Bouin, Ehrlich's haem. and eosin.

6

7

8

9

zona fasciculata

zona reticularis ("lipoid" free)

medulla ("lipoid" free)

10

zona fasciculata

zona reticularis (with small "lipoid" lobules)

medulla ("lipoid" free)

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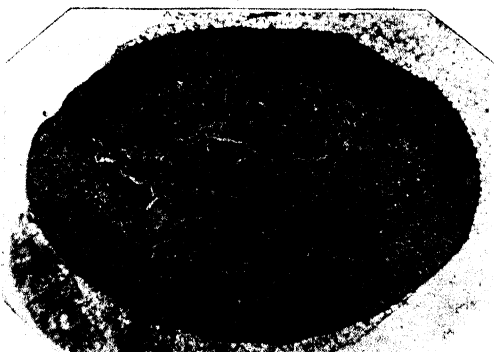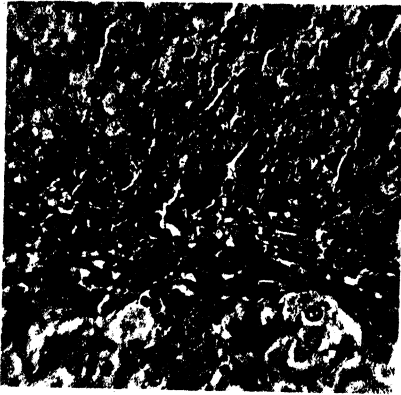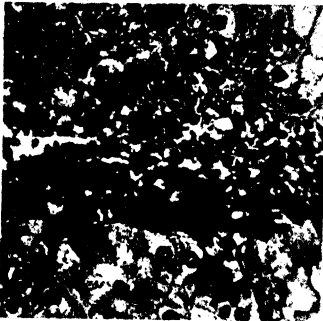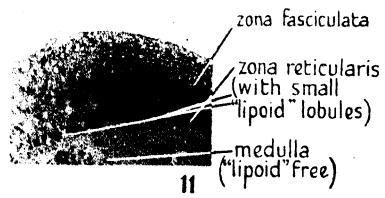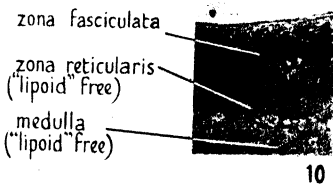
I. CHESTER JONES.—PLATE II

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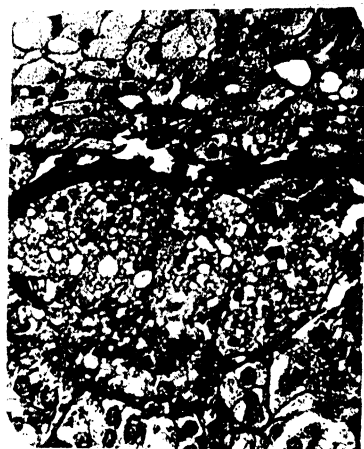


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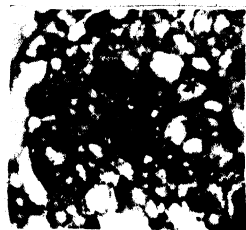


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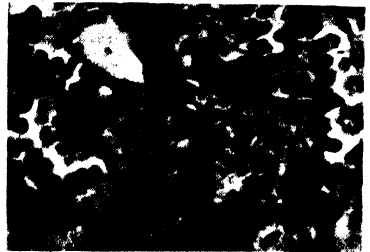
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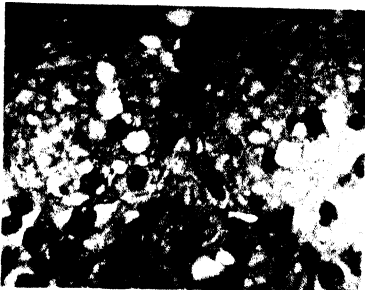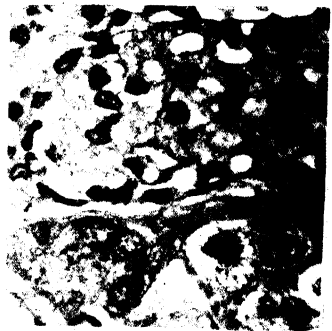
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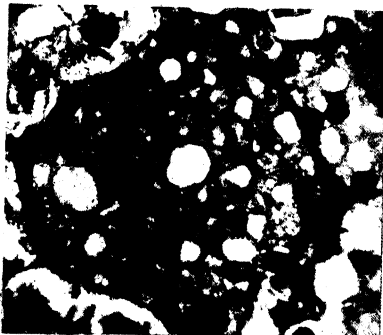
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# Cytology of the Glands associated with the Alimentary Tract of Domestic Fowl (*Gallus domesticus*)

BY

## K. S. CHODNIK

*(From the Department of Zoology, University of Edinburgh)*

**With twenty-nine Text-figures**

## INTRODUCTION

IN a previous contribution the writer described the cytoplasmic components in the cells of the alimentary tract of the domestic fowl during different phases of cell activity (Chodnik, 1947). The present account records similar observations on the salivary glands, pancreas, and liver. So far as the writer is aware there are no accounts of the cytology of these glands in birds other than those on the chick liver (Dalton, 1933; Kater, 1933 and 1937). There are, however, many papers on the cytology of the glands associated with the alimentary tract of other animals.

## MATERIAL AND METHODS

The material was obtained from birds which were used for the study on the alimentary tract; the same methods of fixation and subsequent treatment were employed. Material was fixed after a 24-hours' fast, and at periods from half an hour to 6 hours after a meal. The liver appears to be the most difficult tissue, as regards both penetration and fixation, especially with fluids containing osmium tetroxide. Regaud's technique proved to be the best for mitochondria. In the pancreas, however, it gave as a rule an uneven fixation of the superficial and deeper parts of the tissue; in the case of this gland Meves's method was preferable. For the study of the secretory process of the salivary glands the useful material was that treated according to Regaud's technique and counter-stained with Southgate's mucicarmine. To obtain a successful preparation of the Golgi material in the liver, it is essential to reduce the osmium tetroxide (after Mann-Kopsch, or preferably after Ludford's modification) in distilled water for at least 2 days at 35–7° C.

## OBSERVATIONS

### Salivary Glands

In the preliminary work single anatomical agglomerations of the glands in the mouth cavity, and samples of glands scattered on the dorsal part of the crop and throughout the entire length of the oesophagus, were dealt with separately. Owing to their similarity all these glands were dealt with in the subsequent work as one unit. As regards the general features of the salivary glands of the domestic fowl it appears that, in contrast to the variety of cells

encountered in the salivary glands of mammals, there is great simplicity and uniformity in avian material, and that the nature of the sceretory material is exclusively mucous (Heindrich, 1907; Calhoun, 1933). The epithelium of the salivary glands consists of a single layer of cylindrical cells. There is considerable variation in the size and shape of the cells depending upon their position and the phase of secretory activity. Mitotic figures were not observed.

The most common type of cell present, after a 24-hours' fast and at any time after feeding, is an elongated cylindrical form with very faintly marked borders. These cells are completely filled with accumulated secretory material which gives a characteristic reaction with the dyes used to demonstrate mucus (mucicarmine, toluidine blue, thionine). A small spherical nucleus lies close to the basal membrane. The accumulated secretory material is in the form of vesicles, probably of a semifluid consistency, which are divided from each other by narrow cytoplasmic strips joined together and forming a reticulated structure (Text-figs. 8–10). Mitochondria and Golgi material are distributed inside the cytoplasmic strips. Feeding induces very little change in the majority of cells, but after a meal a certain number, which is usually very limited, discharge their content into the glandular lumen where it may be easily demonstrated. Due to the limited number of such cells, very careful examination of the sections is necessary in order to follow the consecutive phases of secretion. It was observed that when a cell is stimulated to discharge its contents the process of evacuation, which commences with the rupture of the cell membrane next to the lumen, continues until the cell is completely empty. Following the evacuation of the secretion, the cell becomes narrow and small, and before it enters upon a new phase of activity, passes through a transitory regenerative phase. It enlarges gradually and refills with cytoplasm. The nucleus moves slowly from the basal part towards the middle region of the cell. Having accomplished its regeneration and the reorganization of all the cytoplasmic components the cell is ready again to enter upon a period of secretion. In the early phase of secretion small mucous vesicles appear in the cytoplasm in the region between the nucleus and the lumen (Text-fig. 7). The vesicles increase in number and size and at the same time move towards the glandular pole of the cell, where a vesicular area of secretory material is formed (Text-figs. 8 and 9). This area expands gradually towards the basal part of the cell displacing most of the cytoplasm. The remaining cytoplasm forms narrow strips which divide and separate the mucous mass into large vesicles. During the accumulation of the secretory material the nucleus moves towards the basal part of the cell. The descent of the nucleus, the non-disappearance of the cytoplasmic components, and the general lack of mitotic figures, suggest that the cell does not disintegrate but regenerates after a secretory cycle and again becomes active. Narrow cells seen among the young cells are probably in the earliest stages of regeneration (Text-fig. 11). The early secretory phases are described in the section dealing with the Golgi material.
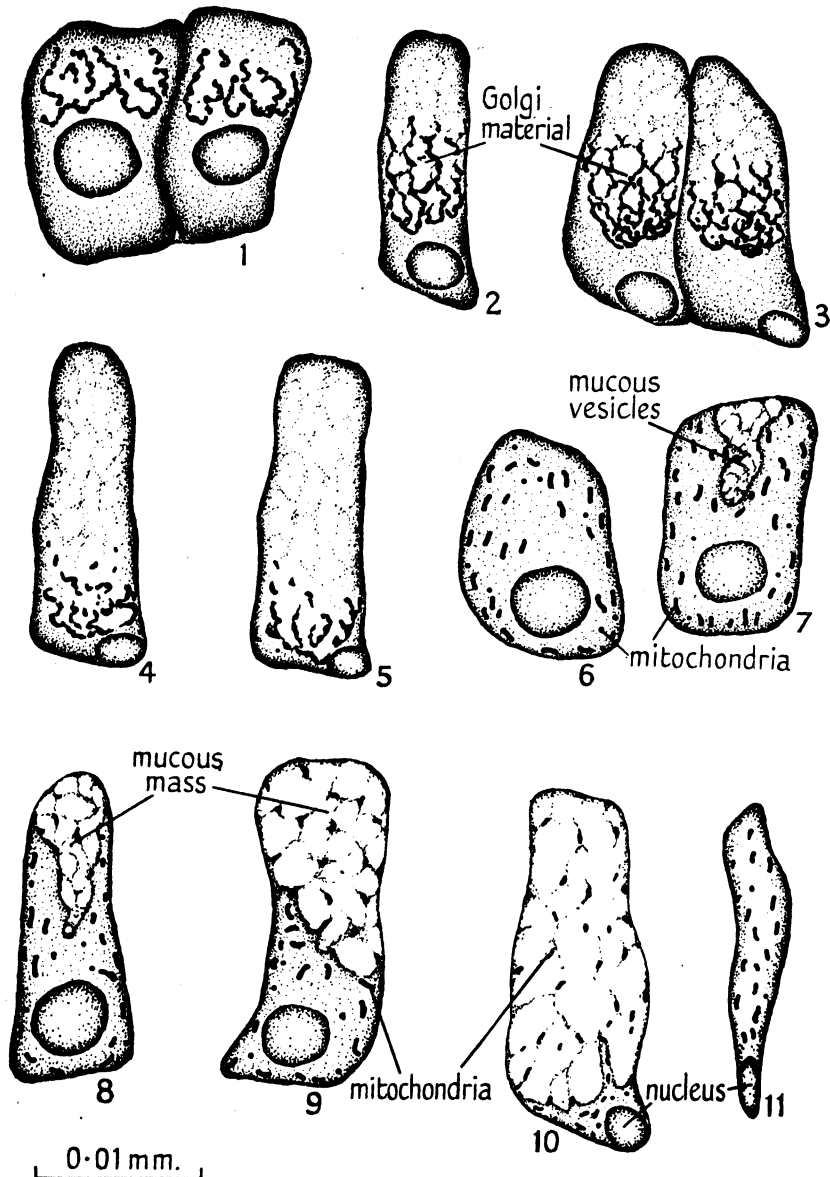
TEXT-FIGS. 1-11. All figures from salivary glands.

FIGS. 1-5 from Kolatchev preparations, showing Golgi material.

FIGS. 6-11 from Champy-Kull and Regaud preparations, showing mitochondria.

Fig. 1.—Regenerated cells before entering upon secretion, Golgi material hypertrophied. Fig. 2.—Cell with secretory granules along the Golgi threads, mucous vesicles surrounded by the ramifications of the Golgi material. Figs. 3 and 4.—Cells in advanced stages of secretion. Fig. 5.—Cell in resting stage, totally filled with mucous secretion, Golgi material reduced in amount. Fig. 6.—Regenerated cell, showing mitochondria. Fig. 7.—Cell in early stages of secretion; mucous vesicles are formed. Figs. 8 and 9.—Cells in advanced stages of secretion, mucous mass expands. Fig. 10.—Cells in resting stage, totally filled with secretion. Fig. 11.—Cell after extrusion of secretory material.

*Golgi material*

The reduced osmium in the Golgi region is in the form of a network. It undergoes marked morphological changes correlated with the different phases of secretory activity. In a dormant cell with the maximum accumulation of secretion, the Golgi material is often difficult to impregnate, and consists of a few threads with small thickenings. It lies quite close to the basal membrane and to the nucleus. Some of the threads follow the cytoplasmic partitions between the secretory vesicles (Text-figs. 4 and 5). Parallel with the regeneration of the cell the Golgi material increases in amount and shows a distinct tendency to spread over a large area always above the nucleus (Text-figs. 2 and 3). In the fully regenerated cell the Golgi material is a strongly hypertrophied structure consisting of lamellar spirals, branched threads, and various short rods and granules (Text-figs. 1 and 2). With the accumulation of the secretory product the Golgi material is gradually pushed towards the basal part of the cell. At the same time its size and power to reduce osmium tetroxide diminish. Finally, it returns to the barely perceptible form with which this description started.

Numerous small granules are present along the faintly outlined threads of the hypertrophied Golgi material of the regenerated cell, particularly in cells prepared by the Mann-Kopsch technique. These are, no doubt, primary secretory granules which later increase slightly in size. Some of the larger granules assume a vesicular form and their power to reduce osmium tetroxide decreases. Later the vesicles no longer reduce osmium tetroxide and now possess an affinity for the dyes used for the demonstration of mucus. Unfortunately it was not possible to follow all the stages of the transformation of the primary secretory granules into mucous vesicles. The larger secretory vesicles are surrounded by the ramifications of the Golgi material which separate them into clusters (Text-figs. 2 and 3). With the accumulation of more material the vesicles tend to fuse and disrupt the outer links of the Golgi material, which moves towards the basal and lateral cell boundaries.

*Mitochondria*

The mitochondria of regenerated cells which have not yet become active are present as numerous rods, long filaments, and granules. Granules are most numerous in the basal subnuclear part of the cell, while rods and filaments predominate in other parts of the cytoplasm and are more or less evenly distributed. Both rods and filaments are arranged parallel to the long axis of the cell (Text-fig. 6). In older cells, under the pressure of the secretory material, the majority of the mitochondria retreat to the cytoplasm in the basal and peripheral regions; a few, however, are scattered in the cytoplasm between the vesicular areas (Text-figs. 9 and 10). In the cells totally filled with secretion, granules and short rods are the prevalent forms. It is difficult, however, to determine if there is an actual decrease in the number of mitochondria present during the dormant phase as many small forms situated between the mucous masses may easily be overlooked. Although a few mitochondria are

usually scattered on the periphery of the mass of secretion, there is no evidence that they give rise to secretory material.

### Pancreas

The following account refers to the acinar cells; the cells of the islets of Langerhans were not studied. In spite of the ease with which good cytological preparations were obtained, and the large size of the cells, the acinar cells are difficult to study when food is used to stimulate the secretory processes. The cells composing an acinus are of an irregular pyramidal shape. A large spherical nucleus lies near the basal membrane or close to the middle region of the cell depending upon the functional stage. In the normal resting phase, subsequent to a 24-hours' fast, the spherical secretory (zymogenic) granules totally fill the portion of the cell between the nucleus and the lumen of the acinus. They vary considerably in size and staining properties. The smaller granules usually stain deeply with acid fuchsin, while the larger ones stain a very faint pink or lose their affinity for acid fuchsin and take on a yellow colour from the picric acid used in differentiation. Granules very rarely extend to the extreme basal part of the cell. Soon after feeding (half an hour) a large number of secretory granules are evacuated, but total evacuation was never observed. In many cells, unstained vacuoles mark the position formerly occupied by secretory granules.

The present observation confirms previous statements (Hirsch, 1932; Ries, 1935) that the pancreatic acinus acts as an autonomous unit. Some of the acini, fixed half an hour after feeding, contain very few granules, while others may contain a large number and remain in an unchanged condition similar to the resting phase. It was not possible to follow the order in which the evacuation of the granules takes place. The release of the granules, which begins soon after feeding, continues for about 2 hours after the intake of food. It appears that as soon as the discharge of secretion begins, the formation of new granules takes place. Numerous small granules become visible in the supranuclear zone. These early secretory granules stain very deeply with acid fuchsin, in contrast to the older granules, which take on a faint stain. Granules were observed in fixed material, in material stained supravitally with neutral red, and in unstained cells in which they appear as highly refractile bodies. Two hours after feeding, there is great variation between the different acini; some are entering upon the secretory phase while others contain large secretory granules situated between numerous small ones. The process of restitution progresses gradually to the stage observed after a 24-hours' fast, and terminates about 5–6 hours after feeding. In spite of a careful search of fixed and stained preparations granules were not observed in the subnuclear zone, but small granules were visible in this region of cells which were stained supravitally.

### Golgi material

The Golgi material was identified in acinar cells during a fast, when there is a maximal accumulation of secretory granules, and at each stage subsequent

to feeding. It appears to form a support and framework for a substance which reduces osmium tetroxide slightly more vigorously than the rest of the cytoplasm (Text-fig. 12). Marked changes were noted in the Golgi material during the production of secretion. After a 24-hours' fast the Golgi material forms a compact, sharply outlined body which lies in the supranuclear region surrounded by the secretory granules. The first visible morphological changes are evident 1 hour after feeding, when there is a loosening of the osmiophilic threads and the field covered by the Golgi material is considerably larger than during a fast. Small sharply outlined swellings are present on the osmiophilic threads. The substance which fills the spaces between the osmiophilic framework reduces osmium tetroxide more vigorously than during the inactive phase (Text-fig. 13); later, there is a further increase in its reducing power. Two hours after feeding the osmiophilic threads become less clearly outlined, and numerous granules are now visible along their course (Text-fig. 14). In the next phase, about 3 hours after feeding, the osmiophilic threads on the glandular side of the Golgi material appear to lose their continuity and secretory granules possessing a narrow osmiophilic rim move away from the Golgi field (Text-fig. 16), and proceed towards the extreme glandular pole of the cell. In about 5–6 hours after feeding the Golgi material almost regains the form and properties characteristic of the resting stage.

*Mitochondria*

The mitochondria of the acinar cells of the domestic fowl are of a similar pattern to that described by most workers on the pancreas of other animals. They are remarkable for their size and length. A great variety of forms are encountered in each cell—long filaments, often extending for two-thirds the length of the cell, short rods, and granules. The filaments are generally slightly tortuous. All the mitochondria appear to lie for the most part parallel to the long axis of the cell, but some may lie parallel to the basal membrane (Text-figs. 19 and 20). In the osmium fixed material the presence of secretory granules renders the examination of the mitochondria in the supranuclear zone difficult, and often impossible (resting stage). In material fixed and stained according to Regaud's technique, the mitochondria are clearly visible in all parts of the cell (Text-fig. 19). The long filaments are commonly seen along the lateral cell walls. At the time of increased cellular activity, long tortuous forms are very seldom seen and more regular rods prevail. This is most notable 2–3 hours after feeding.

*Liver*

Dalton (1933) gave a thorough description of the changes in the cytoplasmic components of the cells of the liver of the chicken from the earliest embryonic stages to the time of hatching and also a short account of the changes caused by fasting and feeding. He stated that, although the mitochondria and Golgi material assume characteristic patterns correlated with increased and decreased cellular activity, there is no evidence of a morphological character which
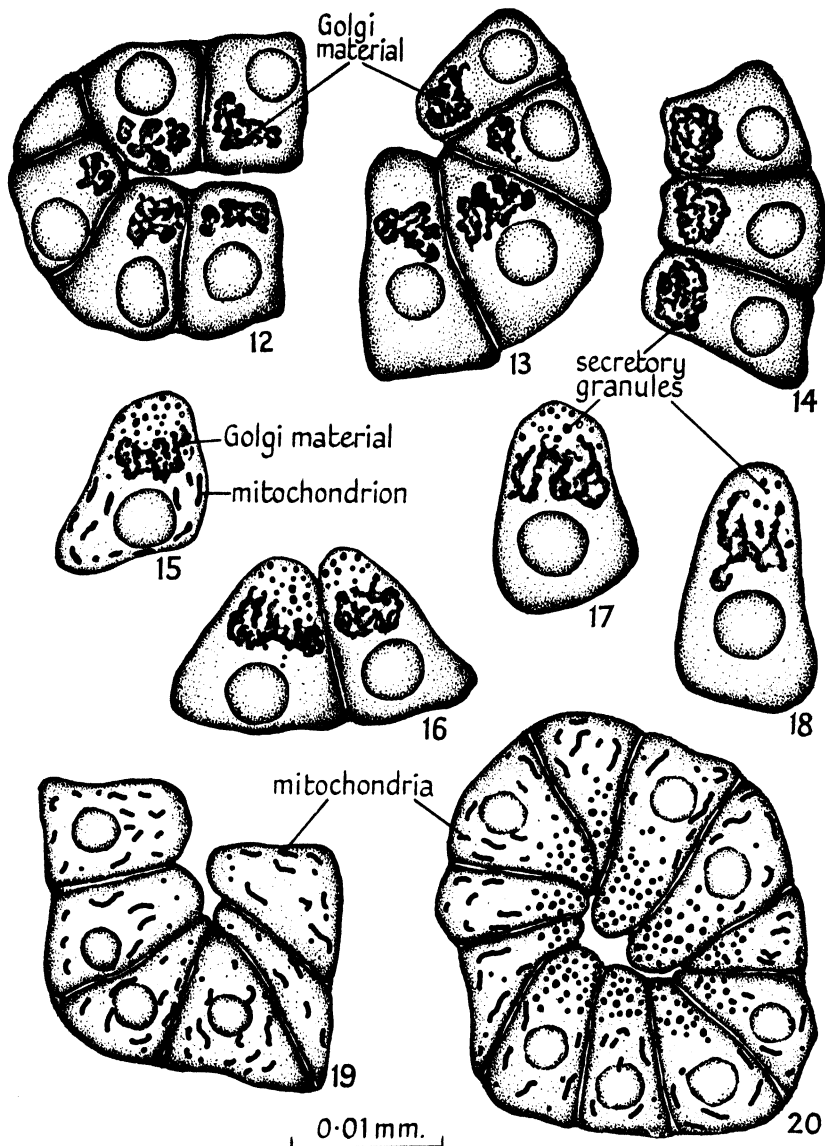
TEXT-FIGS. 12–20. All figures from pancreas.

FIGS. 12–18 from Kolatchev preparations, showing Golgi material.

FIG. 19 from Regaud preparations, showing mitochondria.

FIG. 20 from Meves preparations, showing mitochondria and secretory granules.

Fig. 12.—Cells after a 24-hours' fast. Fig. 13.—Cells 1 hour after feeding. Fig. 14.—Cells 2 hours after feeding, formation of secretory granules. Fig. 15.—Cell 2 hours after feeding, showing Golgi material, mitochondria, and secretory granules. Fig. 16.—Cells 3 hours after feeding, secretory granules accumulate on the glandular pole. Figs. 17 and 18.—Cells from birds with constant access to food, pictures as in advanced stages of secretion. Fig. 19.—Cells showing mitochondria. Fig. 20.—Cells showing mitochondria and secretory granules.

would indicate a specific action of the mitochondria, or the Golgi material, in secretion. During the present investigation, which is an extension of Dalton's work, some interesting results were obtained.

The liver of the domestic fowl is much simpler than that of mammals, and has no true lobular structure. The liver epithelium surrounds the tubules which build the intercellular bile canaliculi. The hepatic cell is an irregular polyhedral pyramid with its apex bordering on the lumen of the bile canaliculus and the basal part in contact with the blood capillaries. The large spherical nucleus lies in the basal part of the cell, and a few globules of fat are usually seen in the surrounding cytoplasm. A thorough search did not reveal any interlobular variations whatsoever. In this paper no reference is made to the glycogen–glucose equilibrium or to the secretion of bile.

### Golgi material

Until the works of Cramer and Ludford (1927), who successfully impregnated the Golgi material of certain mammals, nothing was known about this component of the liver cell. During the present work a comparison of material fixed after a 24-hours' fast with preparations from birds killed at hourly intervals after feeding showed that the form of the Golgi material is very closely correlated with the phases of secretion. In the cells of fasting chickens the Golgi material is located comparatively close to the nucleus. It consists of somewhat thick sausage-like rods, with a few threads between them. These rods are of fairly uniform thickness and are loosely placed in the form of an umbrella above the nucleus (Text-fig. 21). The first changes in the Golgi material were perceptible 1 hour after the intake of food. The thick Golgi rods elongate slightly, become thinner, and arrange themselves above the nucleus like the rays of an open umbrella. At this phase the connecting threads are no longer present (Text-fig. 22). Two hours after a meal, the long rods become more twisted and are again connected with one another by threads, thus forming a considerably more complicated structure. Thickenings and a few granules are visible along the Golgi threads; these react to osmium tetroxide in the same manner as the threads themselves (Text-fig. 23). Four hours after feeding, the Golgi material expands considerably and numerous granules, arranged in rows, replace many of the links. It often appears as though the threads are split into thinner threads with a large number of granules intimately connected with them. At this time most of the granules are small and deeply blackened while the connecting threads are much fainter in outline (Text-figs. 24 and 25). Six hours after feeding, some granules are situated beyond the Golgi field in the form of large vesicles which are very faintly outlined in osmic preparations. The thick threads are replaced by grey lamellae which spread between the small dark granules. The substance between the Golgi links and the granules reduces osmium tetroxide strongly and takes on a darker colour, thus outlining the whole Golgi field (Text-figs. 26 and 27). In the hepatic cells of birds which have constant access to food the Golgi rods are thicker and more deeply impregnated, but are similar in
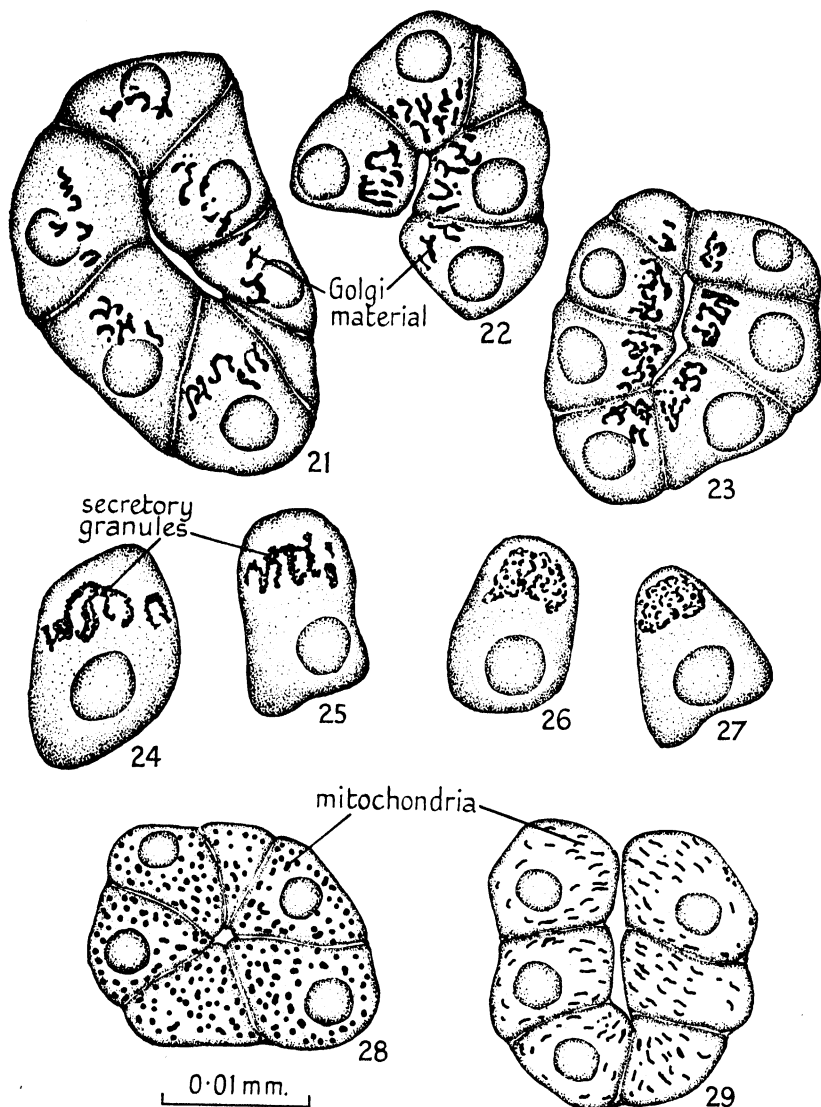
TEXT-FIGS. 21–9. All figures from liver.

FIGS. 21–7 from Ludford preparations, showing Golgi material.

FIGS. 28–9 from Regaud preparations, showing mitochondria.

Fig. 21.—Cells after a 24-hours' fast. Fig. 22.—Cells 1 hour after feeding. Fig. 23.—Cells 2 hours after feeding, enlargement of the Golgi material. Figs. 24 and 25.—Cells 4 hours after feeding, secretory granules seen along the Golgi links. Figs. 26 and 27.—Cells 6 hours after feeding, strong hypertrophy of Golgi material, secretory granules in Golgi field. Fig. 28.—Cells after a 24-hours' fast showing mitochondria. Fig. 29.—Cells 2 hours after feeding, showing mitochondria arranged parallel to the long axis of the cells.

shape to those of the resting stage. In this condition a few granules between and along the thick threads and rods may generally be observed. The whole secretory cycle in the liver cell extends over a period of 6 hours after the intake of food. This is the longest cycle observed in the tissues of the fowl and renders the liver a useful organ in which to follow the consecutive phases. The first secretory granules appear in intimate association with the Golgi material. In the later stages their number increases considerably, but they continue to remain for some time closely connected with the Golgi material and are linked together into an organized body by the osmiophilic lamellae (Text-figs. 26 and 27). The subsequent freeing of the granules from the Golgi field is analogous to the process which takes place in the pancreas.

## Mitochondria

The mitochondria of the hepatic cells show more striking changes during increased cellular activity than in any of the other gland cells investigated by the writer. After a 24-hours' fast, the mitochondria are in the form of granules, ovoid bodies, and short rods. Granules and ovoid forms are most numerous and are present almost exclusively in the basal part of the cell. Rods inter-mingled with granules are seen in the supranuclear zone. The supranuclear mitochondria tend to be arranged more or less parallel to the long axis of the cell, while those in the subnuclear region are scattered at random ('Text-fig. 28). A striking change in the morphological pattern commences 1 hour after feeding and is very prominent after 2 hours, when only a few granules are present while rods and filaments are the predominant forms. The mito-chondria appear to be more numerous, much thinner, and slightly wavy; they have a typical polar orientation from the basal to the glandular pole, and are more or less equally distributed throughout the cytoplasm (Text-fig. 29). From 2 hours after feeding no further change in the mitochondria takes place, but in many cells light irregular areas free of mitochondria appear round the nucleus. The mitochondria are pushed aside and accumulate on the border of these irregular areas, which give a fenestrated appearance to the cytoplasm around the nucleus. These areas correspond closely with those occupied by glycogen which accumulates some hours after feeding. In the material taken 4–5 hours after feeding and fixed by Meves's technique numerous small granules were seen near the glandular pole of the cell and in the Golgi field. The mitochondrial picture of birds with constant access to food does not show any marked difference from that observed in the later hours after feeding. Filaments are predominant, but rods appear to be more numerous than in the early phases of digestion.

<center>DISCUSSION</center>

## Salivary glands

The secretory cycle in the salivary glands of birds resembles in many respects that of the salivary glands of *Tipula paludosa* (Gresson, 1937). There appears to be a similarity in the formation and evacuation of the secretion

and in the regeneration of the cell after each secretory cycle. Unlike the work on the salivary gland cells of insects the present investigation shows a close relationship between the secretory process and the changes undergone by the Golgi material. In that respect the present material appears to be more suitable for determining the relationship between the cytoplasmic components and the secretory product. The strong hypertrophy of the Golgi material in the early phases of secretion, and the presence of numerous granules, which enlarge and become vesicular while in close contact with the Golgi material, are undoubtedly associated with the origin of the secretion of the salivary glands of the fowl. This agrees with previous observations on mucus-secreting cells (Florey, 1932; Duthie, 1933) and is similar to the secretory process observed in intestinal goblet cells (Florey, 1932). Although the mitochondria do not appear to show such a close association with the formation of secretion, as recorded in other gland cells, nevertheless, their arrangement and constant presence on the surface of the mucous vesicles intermingled with the Golgi material indicates that they participate in the process.

*Pancreas*

The acinar cells of the pancreas have been more extensively studied than the cells of any other vertebrate gland. The majority of workers used powerful stimulants (such as pilocarpine) to evacuate the secretion. When food is used as a stimulant to bring about the discharge of the secretion, as it was during the present work, a comparatively small number of secretory granules is evacuated and observation is therefore rendered much more difficult. In spite of these difficulties the consecutive phases of the secretory cycle, accompanied by morphological changes of the cytoplasmic components, were followed with considerable clarity. The behaviour of the Golgi material strongly suggests that it plays an important part in secretory activity.

The present account on the secretory cycle in the acinar cells agrees with earlier descriptions (beginning with Nassonov's paper, 1923) which drew attention to the connexion between the Golgi material and the origin of the granules of secretion. Unfortunately, since fixed material was chiefly used, it was not possible to identify the secretory granules prior to their appearance in the Golgi field. It was therefore impossible to confirm the work of Covell (1928), Hirsch (1932), and Duthie (1933) who stated that there is a connexion between the mitochondria and the early phases of the origin of secretory granules.

*Liver*

The dual role played by the liver cells in the glycogen–glucose equilibrium and the bile secretion and the characteristic lobular structure of the liver has stimulated much research on the function of these cells under different physiological conditions. In particular the function of the mitochondria in the hepatic lobule has been the subject of much speculation. The function of the mitochondria has been ascribed either to carbohydrate assimilation

(McA. Kater, 1931, 1937; Clark and Hair, 1932), or to the secretion of bile (Cramer and Ludford, 1926–7). It appears to be generally accepted that the presence of spherical mitochondria indicates an inactive phase and that the filamentous type is present during secretory activity (Dalton, 1933; McCudry, 1939, 1940; Steffens, 1941). All the earlier workers mention the great technical difficulties involved in studying the hepatic cell, especially in demonstrating the Golgi material. It is almost certain that works based on silver nitrate impregnation must be treated very sceptically, because the most reliable observations have shown that silver nitrate fails to impregnate liver cells (Cramer and Ludford, 1926–7; Pollister, 1932; Dalton, 1933; Subramaniam, 1938). In spite of the difficulties encountered very spectacular morphological changes, both of the Golgi material and mitochondria, were observed during the present investigation. All changes observed in the cytoplasmic components of the liver cell are closely connected with cellular activity and secretory phenomena. The very slow morphological changes observed in the Golgi material render the hepatic cell of the fowl ideal material for the study of the phases of secretion. It is very evident that the granules of secretion appear in close topographical relationship to the Golgi material and that the Golgi material undergoes changes of form and disposition which are correlated step by step with the stages of secretory activity. The present observation on the Golgi material in the liver cell agrees very closely in nearly every respect with Subramaniam's studies on the Golgi material of the liver of *Rhacophorus maculatus* Gray (1938). The morphological alteration of the mitochondria is no less closely connected with the increase of cellular activity. One hour after feeding, the filamentous mitochondria become oriented parallel to the main axis of the cell; this change and the redisposition of mitochondria are much more striking than any which occur in the glandular epithelia of the alimentary tract and other associated glands. Whatever the function of the mitochondria may be, their rhythm depends entirely on the time of feeding, and their response appears to be more rapid than that observed in the liver cells of other vertebrate animals. There is no justification for the conclusion drawn by McA. Kater (1933 and 1937) that the mitochondria show no regularity in the liver of the domestic fowl as regards their morphological features, and that the mitochondrial pattern is so irregular that it can never be regarded as normal. Kater's conclusion can only be attributed to the harmful effect of the stimulants used, or even to the methods of feeding which he employed. The present observation agrees with the generally accepted opinion that granular mitochondria occur during the inactive stage while filaments indicate functional activity.

## SUMMARY

*Salivary glands*

The onset of secretion is marked by hypertrophy of the Golgi material. During the secretory phase the mitochondria lie parallel to the long axis of the cell. Secretory granules are first visible in close association with the Golgi

material. Feeding brings about the evacuation of the secretion. Evacuation of secretion is total; regeneration of the cell precedes the next secretory phase.

### Pancreas

The Golgi material enlarges during the onset of secretion. Few changes were observed in the morphology of the mitochondria. Secretory granules are first visible in close association with the Golgi material. Later they move towards the acinar lumen. Feeding brings about evacuation of the secretion granules, but total evacuation does not take place.

### Liver

The morphology of the Golgi material changes considerably during the production of the secretory granules. Granular mitochondria predominate after a 24-hours' fast. Rods and filaments are prevalent after feeding. During the secretory phase the mitochondria lie parallel to the long axis of the cell. The secretory granules are first visible in association with the Golgi material. Free secretory granules are not visible until 6 hours after feeding.

REFERENCES

CALHOUN, M. L., 1933. Iowa St. Coll. J. Sci., **7**, 261.
CHODNIK, K. S., 1947. Quart. J. micr. Sci., **88**, 419.
CLARK, J. A., and HAIR, G. E., 1932. Z. Zellforsch. mikr. Anat., **15**, 123.
COVELL, W. P., 1928. Anat. Rec., **40**, 213.
CRAMER, W., and LUDFORD, R. J., 1926–7. J. Physiol., **62**, 74.
DALTON, A. J., 1933. Anat. Rec., **58**, 321.
DUTHIE, E. S., 1933. Proc. Roy. Soc. B, **113**, 459.
—— 1934. Ibid., **114**, 20.
FLOREY, H., 1932. Brit. J. exp. Path., **13**, 349.
GRESSON, R. A. R., 1937. Z. Zellforsch. mikr. Anat., **25**, 131.
HALL, E. M., and MACKAY, E. M., 1933. Amer. J. Path., **9**, 205.
HEINDRICH, K., 1907–8. Morph. Jb., **37**, 10.
HIRSCH, G. C., 1931. Biol. Rev., **6**, 88.
—— 1932. Z. Zellforsch. mikr. Anat., **15**, 36, 290.
McA. KATER, J., 1931. Anat. Rec., **49**, 277.
—— 1933. Z. Zellforsch. mikr. Anat., **17**, 217.
—— 1937. Ibid., **25**, 127.
McCUDRY, M. B. D., 1939. J. Morph., **64**, 9.
—— 1940. Biol. Bull., **79**, 252.
NASSONOV, D., 1923–4. Arch. mikr. Anat., **97**, 136; **100**, 433.
POLLISTER, A. W., 1932. Amer. J. Anat., **50**, 179.
RIES, E., 1935. Z. Zellforsch. mikr. Anat., **22**, 523.
STEFFENS, H. W., 1941. Anat. Rec., **81**, 243.
SUBRAMANIAM, M. K., 1938. Proc. Indian Acad. Sci. B, **7**, 80, 125.

# Observations on the Chemical Composition of Myelin and the Smallest Size of Myelinated Nerve-fibres in the Central Nervous System

BY

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**With one Plate**

## INTRODUCTION

THE methods of polarization optics and X-ray diffraction (Schmitt and Bear, 1937, 1939) have shown that the myelin sheath consists of thin sheets of protein, wrapped concentrically about the axon, with two bimolecular layers of lipoids interspersed between adjacent protein layers: there is birefringence due preponderantly to lipoids in the case of larger fibres, graded down to that in smallest fibres resulting primarily from proteins, the transition from lipoid to protein predominance occurring at a diameter of about 2 $\mu$. (The nomenclature used in this paper is as follows: Lipoids, a generic term for all substances extractable from tissues by fat solvents include, amongst others, lipides (lipids) all of which contain at least one fatty acid. These include the triglycerides, esters of fatty acids with other alcohols (such as sterols) and lipines, which are those lipides containing nitrogen and sometimes also phosphorus, i.e. the phospholipines and galactolipines.) There is, however, little information in the literature concerning the histo-chemical constitution of myelin. Bielschowsky (1935) has stated that myelin has a complex structure, containing cholesterol, lecithin (a phospholipine), 'Protagon', and 'Cerebrin'. He also asserts that lecithin is the substance coloured by Weigert's method, although he does not give his reasons for saying so. Page (1937), in his monograph on the chemistry of the brain, states that brain tissue is very rich in phosphatide (i.e. phospholipines), containing about twice as much as liver and kidney and three times as much as heart-muscle, much of it being contained in myelin sheaths, but the cells themselves not lacking it. It is therefore not surprising that 'phosphatides compose a large part of the lipids in brain'. Koch and Koch (1913) determined that the most active period of medullation in albino rats is during the tenth to twentieth days postpartum and that during this period phosphatide formation is the predominant feature.

The current usage of the terms 'myelinated' and 'unmyelinated' may tend to convey the impression that the two designations denote two fundamentally

different kinds of nerve-fibres. However, it appears to have become generally believed, although as far as is known not yet proved, that the distinction is mainly one of a quantitative nature. Thus, from an anatomical point of view, Ranson, Droegemueller, Davenport, and Fisher (1935) state that 'anatomically no sharp line of demarcation can be drawn between myelinated and unmyelinated fibres'. The fact that fibres of the same category may be unmyelinated in smaller animals and myelinated in other, larger, animals (e.g. the fibres of the ventral and dorsal roots as shown by Duncan, 1932, 1934) strongly supports this contention. Kiss and Mihalik (1929) from anatomical studies conclude that even the smallest nerve-fibres of the spinal nerves and roots have a myelin sheath, although it appears that the evidence on which their statement rests is somewhat equivocal. The findings in electrophysiological studies of nerve-fibre conduction do not lend support to the assumption of fundamental differences between fibres which are myelinated and those which are commonly regarded as being unmyelinated (see e.g. Blair and Erlanger, 1933; Grundfest and Gasser, 1938). By workers on nerve conduction it is commonly stated that the fibres of lowest conduction velocity, the C fibres, are mainly, at least, unmyelinated fibres (see e.g. Zotterman, 1939), and the two terms are sometimes used synonymously.

It appears that the lowest limit of the thickness of myelinated fibres is mainly a question of the methods applied in their study. According to the literature the finest myelinated fibres have been observed in osmium tetroxide preparations, which allow the tracing of somewhat finer fibres than the Weigert technique and its modifications. Several authors describe myelinated nerve-fibres in the spinal roots and peripheral nerves in osmium preparations of a diameter of $1-2\mu$ (see e.g. Table 1 in Duncan, 1934; Foley and Dubois, 1943; Rexed, 1944, and many other authors). Some have even observed fibres less than $1\mu$ in thickness (e.g. Duncan, 1934, in the pigeon; Hillarp and Olivecrona, 1946, a small proportion in some cases in the cervical sympathetic trunk in the rat). Using polarization optics and X-ray diffraction methods, Schmitt and Bear (1937, 1939) have succeeded in demonstrating that fibres of diameters less than $2\mu$ possess a lipoid investment, its thickness decreasing with the thickness of the axons. However, the proportion of protein to lipoid appears to increase gradually with the fineness of the fibre, and it appears that the finest fibres, less than $1\mu$, cannot be satisfactorily studied by this technique, according to Schmitt and Bear.

In view of the data reviewed above, it was considered worth while to study the application of the histochemical method for detection of lipine worked out by Baker (1946) to the central nervous system. This method, which is claimed to be specific for phospholipines (Baker, 1947), has been applied by Baker to several tissues and has been used by Harrison and Cain (1947) in the study of the adrenal cortex. The present study was undertaken with the following aims in view: first, to gain information concerning the chemical constitution of myelin, and secondly, to determine the possibility of tracing myelinated fibres thinner than those revealed by other methods.

The material used for this investigation consisted of human and rats' brains. The human brain was obtained from an individual not known to have suffered from any neurological disorder.[1] The rats' brains were from adult male rats. In addition to the use of Baker's method, other sections were treated with sudan black (see Baker, 1944) or nile blue (see Cain, 1947 *a* and *b*). The acid haematein test of Baker (1946) is a modification of the Smith-Dietrich test, used in conjunction with a pyridine extraction control test. A phospholipine is recognized by a blue or blue-black colour seen after acid haematein but not after pyridine extraction. A blue colour after pyridine extraction is due most commonly to acidic proteins but may, of course, be due to lipines not completely extracted. These acidic proteins also stain after acid haematein. The thickness of the fibres was measured by means of an eye-piece micrometer with oil immersion.

OBSERVATIONS

A. *Acid Haematein-stained Material*

*Rat Material*

In sections of the central nervous system of rats stained by the acid haematein test the myelinated fibre tracts and the white matter of the cerebrum and cerebellum are seen with the naked eye to acquire a pure blue tint as in a Weigert-Pal preparation. The grey matter appears grey-blue. On microscopical examination this is observed to be due to the staining of numerous fibres within the grey matter. The intensity of the colour depends on the thickness of the section. Thin sections, $6-10\mu$, are best suited for the study of details and were used in this investigation. Baker (1946) states that sections $10\mu$ or less in thickness must be used; while thicker sections might well introduce sources of error, thinner ones do not lose in specificity by slightly heavier differentiation. The findings made on examination of various parts of the central nervous system of the rat will be described below.

*Cerebral Cortex.* With the low power a fair number of coarser radiating fibres can be followed through all cortical layers into layer II. In addition some finer radiating and a certain number of tangential and oblique fibres are seen. When the sections are studied with higher power or with oil immersion, a multitude of finer and coarser blue streaks and minute blue spots and rings interspersed between the cells and coarser fibres are recognized (Pl. I, fig 1). The coarser fibres appear blue-bordered on both sides, as would be the case if the myelin sheath and not the axon were stained. That the blue stain is not taken up by the axon itself is shown by the pictures of transected coarser fibres, which present themselves as blue rings with no coloration of their central areas. The smallest fibres which possess a double-bordered outline have an outer diameter of about $1\mu$, and correspondingly the smallest rings are of the same diameter, some even somewhat smaller. In

[1] A male aged 61, death occurring on account of coronary occlusion.

the latter the blue ring surrounding the central pale area is very thin. In addition to these fibres and rings, which are clearly to be interpreted as nerve-fibres endowed with a myelin sheath, there occurs, however, a multitude of finer blue lines and streaks, which even with the highest magnification cannot be seen to present a double blue border-line (Pl. I, fig. 2). Some of these pursue a fairly straight course for some distance, but the majority of them make frequent bends and can consequently in these thin sections be followed as uninterrupted structures only for a relatively short distance. Occasionally they may be seen to branch dichotomously, or to give off side branches, presumably collaterals. Now and then it is also possible to follow the gradual transition of one of the smallest double-bordered fibres into a very thin singly contoured fibre of a diameter of about $0.5\,\mu$. Quite frequently these show smaller swellings, being for a short distance double-bordered, or showing small heavier blue spherules along their course (see Pl. I, fig. 2). The meshes between the nerve-cells of the cortex are partly filled with fibres, predominantly of the smallest calibre and minute rings (cross-sectioned fibres), but in addition there are numerous minute granules, and very short fragments of the tiniest blue fibres. The distinctly outlined minute blue spots are presumably cross-sectioned fibres of the finest type. Their diameter is between 1 and $0.5\,\mu$, some even less, but definite estimation of the thickness is impossible with smaller diameters. The individual nerve-cells are frequently seen to be encircled by some such extremely fine fibres.

The nerve-cells are usually not stained, and have a very faint yellow-white colour. The nucleus is visible when the opening of the diaphragm is reduced, as is also the nucleolus. In some places, however, some of the nerve-cells are shrunken, with definite angles, and have either a dark, dirty yellow colour, or are stained blue-black. These changed nerve-cells correspond to the artificially shrunken cells, which are commonly observed in the brains of animals, mainly near the surface, and which have been studied by several authors. By some their appearance is assumed to be due to mechanical interference in the dissection of the brain, a view which would explain their tendency to occur in superficial parts of the brain and in patches (see e.g. Droogleever Fortuyn, 1927; Scharrer, 1937 and 1938). In some of these cells the apical dendrite, and in others the neurite, can be seen to have acquired a blue colour. The connective tissue of the meninges and blood-vessels is slightly yellow, the red blood corpuscles being a blue to dark blue-black colour.

Mainly similar findings are made in several other parts of the central nervous system of rats, of which only some will be mentioned below.

*The Cerebellum.* The cortex assumes a bluish colour. When seen with high power, however, there is in most places no clear-cut pattern of the fibres in the molecular layer as seen in silver preparations, but the blue tint is observed to be due to a limited extent only to some very tiny fibres of about $0.5\,\mu$; some of these ascend towards the surface, others are horizontal. In addition very fine blue dots occur everywhere. In some places, however, mainly in superficial parts of the cerebellum, where the Purkinje cells are shrunken (cf. below), a

multitude of extremely thin tangential fibres is visible (in longitudinal sections of the folia, and therefore mainly neurites from granule cells). Only very few rings of the smallest type are observed, as evidence of cross-sectioned myelinated fibres. That the finest fibres are not glial fibres is extremely probable from the occurrence of swellings and irregularities in their calibre which are well known to occur in preparations of myelinated fibres. Just as in the cerebral cortex, it may be observed how a single contoured fibre of a diameter of about $0.5\mu$ for a short distance is swollen and shows a double-bordered part, similar in appearance to the thicker myelinated fibres elsewhere. In the granular layer mostly coarser fibres are seen, but quite a number of smaller ones, usually of an irregular course or transected, are to be found (Pl. I, fig. 5). Between groups of granular cells accumulations of small blue dots occur interspersed with some distinguishable fine fibres, corresponding, it appears, to the so-called cerebellar glomeruli. It is not possible to decide whether these blue patches are only conglomerations of stained fibres or precipitation artifacts in the matrix (though neither formal calcium nor dichromate calcium is a protein precipitant). Many of the Purkinje cell nuclei are shrunken and stain more or less blue-black. Such cells are found mainly in the superficial folia, less in the depths of the sulci. In some of these cells also the branching dendrite can be followed (and in the same regions the amount of finest fibres is particularly large). It is, however, striking that the fibre baskets surrounding the Purkinje cells and the supra- and infraganglionic layers of tangential fibres so clearly seen in silver preparations are nowhere visible, only a few medium-sized fibres occurring in these places. The picture of the intracerebellar nuclei is clearer; coarser, finer, and finest fibres, longitudinally or transversely cut, occur in abundance between the nerve-cells. It should be noticed that the cells of the cerebellar nuclei regularly contain a fair amount of fine blue granules in their cytoplasm; these are frequently arranged in rows, like the tigroid granules, but on the whole the size of the granules observed in our preparations is finer and they are of more uniform size than the tigroid granules.

*The Hypothalamus.* This region, commonly regarded as being scanty in myelinated fibres, is distinguished also in the acid haematein preparations by being less blue than most other regions of grey matter. However, with high power, quite a number of blue fibres are found, transversely or longitudinally cut. Coarser fibres are very rare, and the majority of the fibres observed are of the medium and finest type. Also in this region numerous blue fibres occur, with a diameter of less than $1\mu$ down to about $0.5\mu$, and ring-like structures and dots of corresponding size are frequent. The medial forebrain bundle can be identified with the naked eye in these preparations by its bluish tint.

*The Spinal Cord.* Similar small fibres are observed as in the other parts. Thus, extremely fine fibres can be seen entering the grey matter of the dorsal horns and coursing in a ventral direction, some of these having a diameter of about $0.5\mu$, or even less (cf. Pl. I, figs. 3 and 4).

*Human Material*

In principle the findings are similar to those in the preparations from the rats' brains. However, on gross examination, the grey matter appears less blue than in the sections of the latter, and on microscopical examination the explanation of this difference is obvious. The number of finer fibres acquiring a blue colour is definitely less, and in addition the blue colour of the thicker and medium-sized myelinated fibres is distinctly lighter than in the rats. However, there are also present in the sections of the human brain, for instance in those from the cerebral cortex, small blue rings of a diameter of about $1\mu$, and now and then a tiny blue fibre of a diameter of about $0 \cdot 5\mu$ is encountered, revealing the same properties as described in the similar fibres in the rat material. Furthermore, the multitude of minute blue dots and fragments of linear structures, to be seen everywhere in the grey matter of the rats' brains, is almost completely lacking in the corresponding areas of the human brain, where the myelinated, coloured fibres stand out against a pale-yellowish background. In other parts of the brain, as for instance the hypothalmus, the cerebellar cortex, and the inferior olives, the number of blue fibres and dots of the smallest size is even less than in the cerebral cortex and almost negligible. Shrunken cells, assuming a dark-grey or blue colour in acid haematein preparations, are not seen in the human brain, in accordance with their regular absence after the usual methods of preparation.

## B. *Pyridine-extracted Material*

In the sections from the rat the white matter appears grey with a slightly blue tint. The grey matter acquires a yellow hue. On microscopical examination the cells are seen to be yellow, while the nucleolus and sometimes also some of the chromatin material assumes a dark blue-black colour. Since the findings are essentially similar in all regions of the brain, only a general description of the appearance of the fibres will be given.

The coarser fibres, regardless of whether they occur isolated or in larger bundles, have a greyish colour with a slight blue tint. The blue tint is, on the whole, a little more prominent in the deeper regions than in the superficial, a fact which may possibly indicate that the lipoid extraction has been less complete in the deeper parts of the block than in the more superficial or, since extraction in general seemed thorough throughout the block, that these fibres contained either more acidic protein, or a protein–lipine complex from which the lipine could not be extracted. Apart from this change in colour as compared with the acid haematein material, the appearance of the myelin sheaths is more diffuse, they are less clearly seen as individual units, less sharply outlined, and the details in the areas composed largely of myelinated fibres on this account appear somewhat indistinct. In cross-sections of such areas, the myelin sheaths are seen as rings, but these are thinner than when seen in acid haematein preparations, and their outline is also less distinct. Their more irregular shape, sometimes wrinkled, may be due to the pyridine-extraction fixative which is a strong protein precipitant. Quite frequently

small or minute dark-blue spots occur, almost like pearls, along the course of the myelin sheaths, possibly indicating a deficient extraction of lipine or, more probably, that the precipitating action of the fixative has produced granules of protein just as it does in nuclei.

Smaller fibres are also to be seen in fair numbers, a few of them even of the same small size as in the acid haematein sections, down to a diameter of about $0.5\mu$. Likewise rings of a diameter of about $1\mu$ can be encountered. However, these are less distinct than in the acid haematein sections, perhaps to some extent on account of their more greyish colour. When the richness of finest fibres is compared with that seen in the acid haematein sections, it appears that their amount is less in the pyridine-extracted material. This is particularly conspicuous in the cerebral cortex, where comparisons are most easily made. Only occasionally in the pyridine-extracted sections are finest fibres observed in the outer half of the thickness of the cortex, whereas such fibres were found consistently also in the superficial parts of the cortex in the acid haematein material. Similar conditions prevail in the cerebellar cortex. The amount of stained fibres in the granular layer is less, and the molecular layer is practically devoid of any stained fibres. However, in parts of all sections there is a considerable number of clear, blue, small rings in the molecular as well as in the granular layer, of the type interpreted in the acid haematein sections as cross-sectioned fine myelin sheaths. Along the finest visible fibres pure-blue spots also occur quite frequently.[1]

In the pyridine-extracted sections the red blood corpuscles are stained blue-black, and the nuclei of cells of the connective tissue like those of the nervous tissue are conspicuous by their darkly stained nucleoli; sometimes also granules of chromatin material are visible.

As in the acid haematein-stained sections, the nerve-cells in several places are shrunken in the pyridine-extracted material. These shrunken cells are darker, dirty grey or slightly blue. It is worthy of mention that in some of the shrunken Purkinje cells, their dendrites with several arborizations are stained a clear blue and stand out very conspicuously.

The pyridine-extracted material from the human brain is, in principle, similar to the rat material, but the difference between the pyridine-extracted sections and the acid haematein-stained sections is less evident. The myelin sheaths of the coarser and medium-sized fibres are greyish and less heavily

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[1] It should be mentioned that in the sections from one of the pyridine-extracted blocks of a rat brain numerous peculiar blue particles were seen, irregularly dispersed throughout the sections, mainly in the grey matter. These minute particles, mostly having a diameter no larger than $1-2\mu$, are spherical, ovoid, or comma-shaped and occur sometimes in irregular conglomerations, sometimes isolated or only a few together. It has not been possible to identify them with either nucleoli, nerve-fibres, glial fibres or intracellular granules, or any other known structures of the nervous system. No explanation can be offered for their occurrence only in this block. A slight difference in the treatment of this block, although not possible to ascertain at the time when the sections were examined, might possibly have occurred and might explain the discrepancy. Assuming that the methods are specific, as presupposed above, it appears likely that these granules may chemically be of protein nature.

coloured than in the acid haematein sections. However, also in the pyridine-extracted sections a certain number of the finest fibres still stand out by their grey-blue colour, and likewise some minute rings of a diameter of about $1\mu$ are present. A small quantity of fibres of a diameter of about $0\cdot5\mu$ can be ascertained, their number being, it appears, a little less than in the acid haematein sections.

## C. *Sudan Black and Nile Blue Preparations*

In sections of the rat brain stained with sudan black the white matter acquires a grey to grey-black colour, according to the thickness of the section and the abundance of myelinated fibres. The grey matter is slightly grey when viewed macroscopically. On microscopical examination the myelin sheaths of the coarser fibres acquire an appearance essentially similar to that seen when other myelin-sheath stains are employed, the sheaths colouring more or less black. The same applies to the medium-sized myelinated fibres, which appear double-bordered (Pl. I, fig. 6). With oil immersion lens, smaller fibres with a diameter of $1\mu$ or somewhat less are also seen, similar to those observed in the acid haematein sections, and rings of the same diameter occur, but, on the whole, their amount is somewhat less and only a few of the very finest fibres can be recognized. Their nature as nerve-fibres is revealed by the fact that they frequently show small double-bordered parts along their course, representing apparently the commonly observed swelling of the myelin sheath. However, these finest fibres stand out somewhat less clearly than in the acid haematein preparations, partly because the greyish colour offers a less favourable contrast to the background than does the pure-blue coloration in the latter sections, and partly because the acid haematein test is more sensitive. It is difficult to decide whether the numerous tiny grey dots in the grey matter, e.g. in the cerebral and cerebellar cortex, represent extremely fine fibres or fragments of fibres in cross-section or not, but this interpretation is favoured by the observation of small linear grey structures, reminiscent of fibres, which are sometimes seen to arborize.

The nerve-cells are usually unstained, or, particularly in the larger cells, their cytoplasm has a faint greyish hue. The artificially shrunken cells assume a darker grey colour of the cytoplasm, the nucleus being colourless.

In nile blue preparations the more heavily myelinated fibre tracts stand out clearly, owing to their pure, light-blue colour. Examined with the high power of the microscope, the myelin sheaths are seen to take a light-blue stain. As in the other preparations described above, in the nile blue stained sections very fine fibres can also be seen, but it is difficult to judge their colour when they approach a thickness of $1\mu$.

It should be noted that the cytoplasm of many, although not all, nerve-cells stains distinctly blue. On this account these cells are very conspicuous even when the sections are examined with low power, and in those stained more heavily the nucleus is stained a lighter blue. In the artificially shrunken cells the blue staining of the cytoplasm is very intense, and the nucleus is only

slightly lighter than this. No red coloration was seen anywhere in the central nervous system.

## DISCUSSION

Before interpreting the findings made in this material, attention should be drawn to some data from the literature relevant to the application of histochemical tests to the central nervous system in general. The setting free of phosphorus, probably due to the breakdown of phosphatides, which appears to start immediately after cessation of the circulation of the brain at death (Jungmann and Kimmelstiel, 1929), is probably negligible in the rat material, since the brains were fixed at the most 10 minutes after the animals were killed. In the case of the human brain autolysis may have played a role, however, for there was an interval of 24½ hours between death and fixation. For this reason, the conclusions drawn from our observations will be mainly based on the findings in the rats' brains. According to Weil (1929, 1946) a diminution in the amount of phospholipines, and less of galactolipines, occurs during formalin fixation (the phospholipines being hydrolysed), increasing with the duration of the fixation and the acidity of the formalin solution. The formal calcium employed in Baker's method is designed, however, to eliminate this loss of lipines.

The observations made in this study give some hints concerning the constitution of myelin sheaths. Baker's (1946) acid haematein test is claimed to be specific for phospholipines: therefore one must presume that our findings have demonstrated the presence of this substance in the myelin sheath. The pyridine-extraction control test, while eliminating lipines, increases the tendency of proteins to give a positive reaction. The finest, and even the myelin sheaths of the coarser, fibres in pyridine-extracted material still have a slight blue tint: an obvious explanation for this is that the lipoid extraction has not been complete, although in our material the extraction was exactly performed, but the possibility is still not excluded that the protein in the myelin sheath is being stained. This point will be further discussed later. Sudan black, after fixation in Baker's (1944) formal calcium, colours all classes of lipoids, and it is quite likely that the phospholipine demonstrated by the acid haematein test does not necessarily constitute all the lipoid in the myelin sheath as shown by sudan black; thus it is possible that galactolipines may be present, and therefore no evidence either for or against the statement that galactolipines form one-third of the myelin sheath (Weil, 1946) has been obtained.

According to Cain (1947b), provided a body is known to be lipoid, nile blue can be used to detect acidic lipoids in it; if it consists only of neutral lipoids it will be coloured red, if acidic lipoids (i.e. fatty acids and other lipoids such as lipines, including lecithin and probably other phospholipines which can behave as acids) are present it will be coloured blue. Pending confirmation of Cain's results it can therefore be provisionally assumed that acidic lipoids are present in the central nervous system but, although no red coloration

was seen, the presence of neutral lipoids is not excluded since a red coloration is easily masked by a blue. The blue staining of the myelin sheaths with nile blue can be taken to confirm, at least partly, the observations with the acid haematein test.

The considerations set forth above concerning the chemical constitution of myelin and the reliability of the histochemical tests employed make pertinent some discussion of the question of myelinated and unmyelinated fibres in the central nervous system. In the acid haematein preparations of the rats' brains, fibres staining the typical blue-black colour and having a diameter of as little as $0.5\mu$, or even somewhat less, were seen. Actually this value might be a little higher, on account of the shrinking due to treatment of the tissue, but the percentage of shrinkage is scarcely of a magnitude which fundamentally alters the values as they were measured in the fixed and stained specimens, since formalin causes no shrinkage (see e.g. Rexed, 1944; Baker, 1945) and the usual dehydrating agents are not used in the acid haematein test. These tiny fibres are most clearly seen in the acid haematein sections, somewhat less distinctly and in a smaller amount in the sudan black and pyridine-extracted sections. This might be explained in accordance with the findings of Schmitt and Bear, that the proportion of protein in the myelin sheath increases with decreasing size, but that a certain amount of lipoid is still present in the smallest myelinated fibres. In the acid haematein sections the lipine constituents, or perhaps at times the protein constituents, will contribute to give a blue-black colour with acid haematein. In the sudan black sections only lipoids colour, but the amount of lipoid in sheaths of the smallest fibres may not be sufficient to give a distinct coloration. In pyridine-extracted sections the proteins, possibly together with some bound lipine, are responsible for the staining properties. It seems from these observations, therefore, always providing the acid haematein test is specific, that, since there is lipoid in these small fibres of a diameter of about $1\mu$ as shown by sudan black, and since in all larger fibres lipine, as shown by the acid haematein test, is present, the lipoid in the smallest fibres of about $0.5\mu$ contains some lipine, though this cannot be stated as a direct inference from the results of the acid haematein test, since the protein of the sheath has some tendency to stain blue after pyridine extraction. This, then, would mean that a myelin sheath must be assumed to be present in fibres of a finer calibre than has been shown by previously employed anatomical methods and the methods of polarization optics. The lower limit of the fibre size investigated by Schmitt and Bear appears to be of the order of $1\mu$, smaller fibres being apparently impossible to analyse by their technique, owing to the small radii of these sheaths. Histologically some authors report having observed a few fibres in some places of a diameter less than $1\mu$, as mentioned in the introduction, but such observations, as far as can be seen, appear to be isolated. Usually the limit between myelinated and unmyelinated fibres is drawn at a thickness of $2\mu$, fibres with a diameter below this value being considered as unmyelinated (see e.g. Duncan, 1934). It appears that neurophysiologists generally

accept this limit, and that the recording of electrical changes in fibres of a smaller diameter is beset with great difficulties (see e.g. Zotterman, 1939).

In this study we have confined ourselves to the central nervous system, and the primary object of the investigation was to try to obtain information concerning the chemical composition of the myelin. We have not been able to discover any studies in the literature on the subject of the smallest size of myelinated fibres present in the central nervous system, whereas this problem has aroused great interest with regard to the peripheral nervous system. The most recent work on the calibre of fibres of the central nervous system appears to be that of Szentágothai-Schimert (1941), but this author considers fibres with a diameter of less than $2\mu$ collectively, and no data on the exact size of the smallest fibres present are given. Nor does Häggqvist (1936) give details in this respect in his study of the fibres of the spinal cord of man, although he mentions that a certain number of fibres thinner than $1\mu$ are present. Since, however, Häggqvist employed a modified Alzheimer-Mann technique which according to Rexed (1944) causes considerable shrinkage (some 27 per cent.), his figures cannot be taken to indicate the presence of fibres thinner than $1\mu$ having a myelin sheath. The data for comparison mentioned in the introduction are therefore, as will be seen, all from studies of the peripheral nervous system, and our results do not necessarily imply that the acid haematein test will be able to show the presence of similarly fine myelinated fibres in the peripheral nerves or autonomic system, although this appears likely. It may be mentioned in this connexion that in well-stained Weigert-Pal sections of the central nervous system of man, occasionally a fine myelinated fibre of a diameter less than $1\mu$ can be encountered. The contrast between this finding and the ample occurrence of these and finer fibres in our rats' brains is probably to be explained by a partial disintegration of the lipoids in the human brain between the time of death and autopsy, partly also by the influence of the formalin fixation (cf. above). The scarcity of such fine fibres in the human brain studied by us with the acid haematein test is probably only due to the former factor, since formalcalcium was used for fixation. It is, however, likely that similar fine myelinated fibres are present also in the human brain, but they escape recognition when some time elapses between death and fixation. The lack of the minute blue dots and linear fragments, so universally present in the grey matter of the rats' brains, is in accord with this view, as is also the lighter colour observed in the thicker myelinated fibres in the human brain. In order to test the validity of these assumptions the brain of a rat was fixed $17\frac{1}{2}$ hours after the animal was killed, and stained with acid haematein. These sections are very similar to those from the human brain. Although occasionally a very few of the finest fibres are seen, the multitude of such fibres and minute blue dots present in those rat brains fixed immediately is completely lacking, and the contrast between the two is very striking.

If the observations made in this study are taken to indicate that fibres of a

diameter as small as $0.5\mu$ possess a lipoid investment, a myelin sheath, the range of the myelinated fibres is made to extend over practically the entire fibre spectrum, and the observations lend support to the now generally held view that there is no significant qualitative difference between myelinated and so-called unmyelinated fibres. Even if it is not possible to measure fibres of a diameter of less than $0.5\mu$ with any degree of accuracy, it appears from our sections that there is probably a considerable quantity of even finer, extremely thin fibres of the same type in the central nervous system. The minute blue dots and tiny linear fragments which are seen everywhere in the grey matter of the rat brain, as described above, are probably such fine fibres. That they are glial fibres appears extremely unlikely, since they betray no relation to the glial nuclei and the pictures do not resemble those given by the specific glia staining methods. These observations are most readily made in the cerebral and cerebellar cortex of the rat's brain. This multitude of finest fibres, then, would probably correspond to the so-called neuropile. Whether the interpretation given here is correct remains to be shown by further research, but if so it would mean that even the finest branches are not devoid of a lipoid investment.

However, even if our observations are taken to show that fibres of a diameter of $0.5\mu$ or less have a lipoid investment, they do not prove that this applies to *all* such fibres. The existence of true unmyelinated fibres is, of course, not excluded by our findings. Attention should be drawn to some observations bearing on this question. In some places, particularly in the Purkinje cell layer and molecular layer of the cerebellum, the number of medium-sized fibres seen in acid haematein sections is certainly less than that shown by a successful silver stain, for example, the clear-cut picture of the baskets surrounding the Purkinje cells not being seen in our preparations. They were also not visible in either pyridine-extracted or sudan black sections. Since many of these fibres are not particularly fine, these observations mean either that these fibres contain so little lipoid that they cannot be detected by these methods or that they are virtually unmyelinated fibres. It should be remembered, however, as is well known, that the silver methods are not histochemically specific. The same applies to the Weigert-Pal method, and herein lies its difference from the acid haematein test.

It has been mentioned that in both acid haematein and pyridine-extracted sections of the rats' brains some of the nerve-cells and their processes are shrunken and stained blue while others are unstained. The explanation for this is most probably that those cells and fibres which are stained have a different chemical constitution from those that are unstained. In the regions where these shrunken cells are most abundant the amount of stained fibres is also much more profuse than usual. This does not mean, of course, that such chemical differences are present in life, for they are probably made obvious by local injury to the cells on removal of the brain. The blue staining is due to either protein or protein-bound lipine in the pyridine-extracted sections, and its presence only in some cells may be therefore due to injury to some

cells and not to others with consequent differential cellular fat or protein phanerosis.

## SUMMARY

Baker's (1946) acid haematein and pyridine-extraction control tests, claimed to be specific for phospholipines (Baker, 1947), have been applied to various parts of the central nervous system of rats and man. The sudan black method for the detection of lipoids and the nile blue method for the staining of acidic lipoids have also been used.

The findings are in agreement with older statements in the literature that myelin contains a considerable amount of phospholipines. It was impossible to determine whether galactolipines or neutral lipoids are also present.

In the acid haematein-stained sections finer fibres were seen than when other stains for myelin sheaths are employed. Fibres with a diameter of $0\cdot5\,\mu$ or even somewhat less were stained in various parts of the central nervous system of rats. It is regarded as probable from these findings that fibres down to $0\cdot5\,\mu$ or even smaller possess a lipoid investment. These observations lend support to the now commonly accepted view that the distinction between myelinated and so-called unmyelinated fibres is arbitrary. Some observations are made, however, which indicate that the presence of truly unmyelinated fibres cannot be excluded.

## REFERENCES

BAKER, J. R., 1944. Quart. J. micr. Sci., **85**, 1–71.
—— 1945. *Cytological Technique*, 2nd ed. London (Methuen).
—— 1946. Quart. J. micr. Sci., **87**, 441–70.
—— 1947. Ibid., **88** (in the press).
BIELSCHOWSKY, M., 1935. Allgemeine Histologie und Histopathologie des Nervensystems, in Bumke, O., and Foerster, O., *Handbuch der Neurologie*, vol. i (1), p. 84. Berlin (Julius Springer).
BLAIR, E. A., and ERLANGER, J., 1933. Amer. J. Physiol., **106**, 524–64.
CAIN, A. J., 1947a. Quart. J. micr. Sci., **88**, 151.
—— 1947b. Ibid., **88** (in the press).
DROOGLEEVER FORTUYN, A. B., 1927. J. Comp. Neur., **42**, 349–91.
DUNCAN, D., 1932. Ibid., **55**, 459–71.
—— 1934. Ibid., **60**, 437–62.
FOLEY, J. O., and DuBois, F., 1943. Ibid., **79**, 79–105.
GRUNDFEST, H., and GASSER, H. S., 1938. Amer. J. Physiol., **123**, 307–18.
HÄGGQVIST, G., 1936. Z. mikr.-anat. Forsch., **39**, 1–34.
HARRISON, R. G., and CAIN, A. J., 1947. J. Anat., Lond., **81**, 286–99.
HILLARP, NILS-ÅKE, and OLIVECRONA, H., 1946. Acta Anatom., **2**, 17–32.
JUNGMANN, H., and KIMMELSTIEL, P., 1929. Biochem. Z., **212**, 347–62.
KISS, F., and MIHALIK, PETER V., 1929. Z. Anat. Entw. Gesch., **88**, 112–51.
KOCH, W., and KOCH, M. L., 1913. J. biol. Chem., **15**, 423–48.
PAGE, I. H., 1937. *Chemistry of the Brain*. London (Baillière, Tindall & Cox).
RANSON, S. W., DROEGEMUELLER, W. H., DAVENPORT, H. K., and FISHER, C., 1935. Res. Publ. Ass. Nerv. Ment. Dis., **15**, 3–34.

REXED, B., 1944. Acta Psychiat. et Neurol., Suppl. 33.
SCHARRER, E., 1937. Beitr. z. Pathologie, **100**, 13–18.
—— 1938. Anat. Rec., **72**, 53–65.
SCHMITT, F. O., and BEAR, R. S., 1937. J. cell. comp. Physiol., **9**, 261–73.
—— —— 1939. Biol. Rev., **14**, 27–50.
SZENTÁGOTHAI-SCHIMERT, J., 1941. Z. Anat. Entw. Gesch., **111**, 201–23.
WEIL, A., 1929. J. biol. Chem., **83**, 601–9.
—— 1946. *Textbook of Neuropathology*, 2nd ed. London (Heinemann).
ZOTTERMAN, Y., 1939. J. Physiol., **95**, 1–28.

## EXPLANATION OF PLATE

Fig. 1. Microphotograph from rat's cerebral cortex. Acid haematein stain, × 500. Medium-sized and small myelinated fibres are seen between the cells. Minute fibres and blue dots account for the darkness of the background.

Fig. 2. Detail from cerebral cortex of rat. Acid haematein stain, × 1,000. Some of the finest fibres with a diameter of about $0.5\,\mu$ are indicated by arrows; others, as well as several of the minute dots and tiny fibre fragments, are out of focus (cf. text).

Fig. 3. Microphotograph from the dorsal horn of rat's spinal cord, showing several fine fibres staining in acid haematein sections. Some of the fibres with a diameter of about $0.5\,\mu$ are indicated by arrows. × 500.

Fig. 4. Detail from the centre of Fig. 3, showing the branching fibre with higher magnification, × 1,800. The irregular calibre characteristic of myelinated fibres as seen in histological preparations is visible.

Fig. 5. Microphotograph from the granular layer of the cerebellar cortex of the rat. Acid haematein stain, × 1,000. Coarser, medium-sized, and very fine fibres are seen between the granular cells. Some of the finest fibres, $c.\ 0.5\,\mu$, are indicated by arrows.

Fig. 6. Microphotograph from the vestibular nuclei in a sudan black stained section of the rat's medulla. × 1,800. In addition to thicker myelinated fibres smaller fibres have also taken the stain.
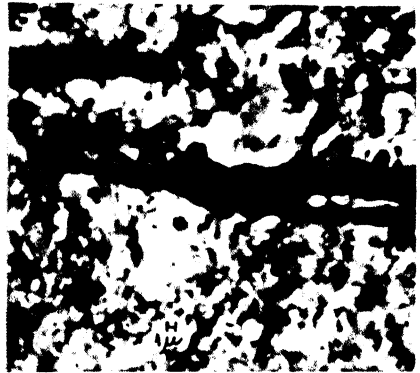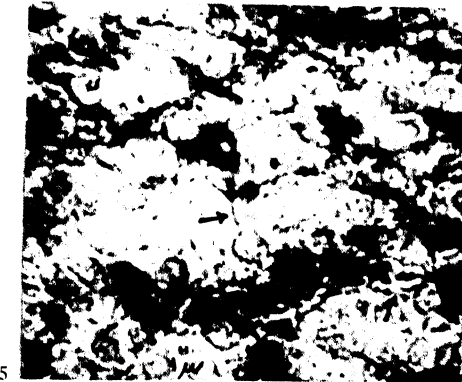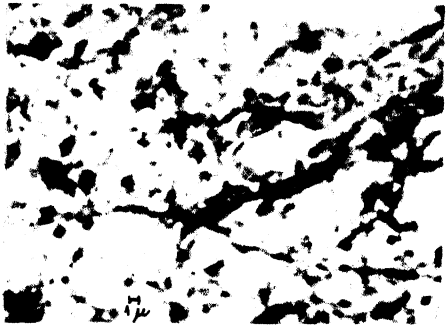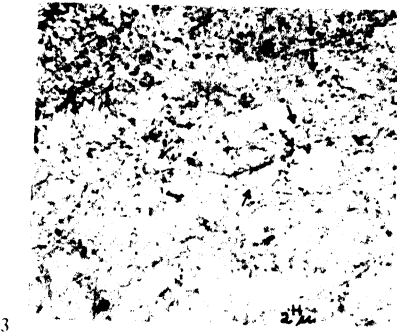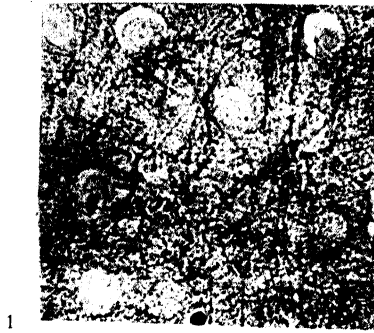
# The Cell-theory: a Restatement, History, and Critique

## PART I

### BY

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### CONTENTS

### INTRODUCTION

SEVERAL zoological text-books published during the last two decades have cast doubts on the validity of the cell-theory. These books do not present in any comprehensible form the evidence on which the doubts are based. I therefore set myself the task of finding and studying the evidence, in order to be able to form some judgement of its weight. When the evidence was examined, it became apparent that different parts of the theory were being attacked, and that one attack might be justifiable while another was not.

Now there is no generally accepted body of opinion as to what the cell-theory is. The phrase 'cell-theory' was invented by Schwann, who has told us what he meant by it (1839*a*, p. 197). 'One may include under the name of *cell-theory*, in the wider sense,' he wrote, 'the exposition of the statement that there exists a general principle of construction (Bildungsprinzip) for all organic products, and that this principle of construction is cell-formation.' Unfortunately, the word Bildungs introduces uncertainty into the meaning of Schwann's definition, for it may refer either to *structure* or to *development of structure*. He seems to have meant the latter. Schwann thought that the development of structure took place in two stages: the development of cells from a structureless substance, and the differentiation of those cells into their definitive forms. He expressed this best in a little-known passage (Schwann, 1839*b*, p. 139), contributed to a work by another author. He here gives a clearer and better definition of the cell-theory than in his celebrated book.

'A common principle of development (Entwickelungsprinzip)', he wrote, 'is the basis of all organic tissues, however diverse they may be, namely, cell-formation (Zellenbildung); that is to say, nature never joins the molecules together directly into a fibre, tube, &c., but always first fashions a cell or first transforms this cell, where necessary, into the different elements of structure as they occur in the adult state.'

Schwann's opinion as to the first stage of the development of structure was quite wrong. In October 1837 he took over from Schleiden, in conversation (Schwann, 1839a, p. x; 1884, p. 25), an entirely false theory as to how cells are formed. Following Schleiden, he called the nucleus a Cytoblast or cell-bud. He thought that nuclei were formed by a process resembling crystallization in a structureless fluid, the Cytoblastem (or cytoblastema, as it is often spelled in English); that each secreted a membrane round itself; and that what we nowadays call the cytoplasm then appeared within this membrane. The method by which cells are formed, however, was not a subject that Schwann investigated with any thoroughness: his research was devoted to the structure of cells, rather than to the process by which they originate. For instance, the cartilage-cell was one of his first objects of study in connexion with the cell-theory (Schwann, 1838a), and he laid especial stress on it in his book (1839a); yet his evidence for the view that it develops from a Cytoblastem in accordance with Schleiden's scheme is wholly indirect. This is made particularly clear in the English edition of his book (1847), for which he rewrote part of the section dealing with cartilage. Nevertheless, Schwann not only believed that animal cells resemble those of plants in their mode of development from a structureless Cytoblastem, but thought that it was he who had discovered this, and insisted on his priority in the matter in the course of an argument with Valentin, attached to the end of his book in both editions.

Schwann drew a sharp distinction between his cell-theory (Zellentheorie) and his theory of the cells (Theorie der Zellen). The former he regarded as inductive, the latter as speculative. In his theory of the cells he is concerned with teleology, with what we should nowadays call the colloid chemistry of cells, and with cellular in relation to organismal individuality. Schwann himself did not regard the last-named subject as part of his cell-theory.

Remak (1855, p. 164), who attributed the cell-theory to Schleiden, defined it as 'the theory of the formation of plants exclusively from homologous components which develop in different ways'. He thus laid stress on the conformity of all plant cells with one another, rather than on the particular way in which they develop. Virchow (1859, p. 9), who was well aware that cells originate from pre-existing cells and who coined his famous aphorism to call attention to this fact, took the phrase 'cell-theory' to mean precisely the erroneous views of Schleiden and Schwann as to their origin.

Other authorities have laid particular stress on the double individuality that they believe to be characteristic of many-celled organisms. Schleiden himself (1838, p. 138) wrote: 'Now each cell leads a double life: the one wholly

independent, only connected with its own development, and the other remote, in so far as it has become an integral part of a plant.' Hertwig (1893, p. 3), in defining the cell-theory, added this idea of individuality to a clause that is reminiscent of Schwann and Remak: 'Animals and plants, so diverse in their external appearance, agree in the fundamental nature of their anatomical construction; for both are composed of similar *elementary units*, which are generally only perceptible under the microscope. Through the influence of an old theory, now discarded, these units are called cells, and thus the doctrine that animals and plants are composed in an accordant manner of very small particles of this kind is called the *cell-theory* . . . the common life-process of a composite organism appears to be nothing else than the exceedingly complicated result of its numerous and diversely-functioning cells.' Bourne (1895, p. 162) also seems to be influenced by Schleiden's ideas when he sums up the cell-theory thus: 'The multicellular organism is a colony, consisting of an aggregation of separate elementary parts, viz. cells. The cells are independent life units, and the organism subsists in its parts and in the harmonious interaction of those parts.' (Bourne himself only accepted a part of this theory as true.) In recent times, Karling (1939, p. 525) has expressed himself similarly: 'The concept of the organism as a mass of cells which integrate and interact to form a co-ordinate whole is perhaps the real climax of the theory.' Other authors have regarded the homology of the cells of many-celled organisms with individual protists as an essential part of the cell-theory. This opinion as to the meaning of the theory has been held by some of those who attack it (e.g. Dobell, 1911). Indeed, the attack on the cell-theory has been mainly directed against this aspect of it.

The diversity of views about the meaning of the expression 'cell-theory' makes it evident that the truth or untruth of the theory can only be established if it can be formulated in clear terms. The different parts of the cell-theory do not necessarily stand or fall together, and the formulation should therefore be in a series of separate propositions, each of which can be examined independently. No proposition which was formerly thought to be a part of the cell-theory, but which is universally discarded by modern biologists, need be included in the formulation; for instance, it would be useless to set up Schleiden's view as to the mode of origin of cells and then demolish it.

Bearing these considerations in mind, I restate the cell-theory in a series of seven propositions, as follows:

    I. Most organisms contain or consist of a large number of microscopical bodies called 'cells', which, in the less differentiated tissues, tend to be polyhedral or nearly spherical.

    II. Cells have certain definable characters. These characters show that cells (*a*) are all of essentially the same nature and (*b*) are *units* of structure.

    III. Cells always arise, directly or indirectly, from pre-existing cells, usually by binary fission.

IV. Cells sometimes become transformed into bodies no longer possessing all the characters of cells. Cells (together with these transformed cells, if present) are the living parts of organisms: that is, the parts to which the synthesis of new material is due. Cellular organisms consist of nothing except cells, transformed cells, and material extruded by cells and by transformed cells (except that in some cases water, with its dissolved substances, is taken directly from the environment into the coelom or other intercellular spaces).

V. Cells are to some extent individuals, and there are therefore two grades of individuality in most organisms: that of the cells, and that of the organism as a whole.

VI. Each cell of a many-celled organism corresponds in certain respects to the whole body of a simple protist.

VII. Many-celled plants and animals probably originated by the adherence of protist individuals after division.

In considering the evidence for and against each proposition, it was necessary to discover how the various bodies of opinion originated and developed. The history of the cell-theory has been told many times, not only in the indispensable standard text-books of the history of biology, but also in other books and in papers. The historical studies that have been of particular use to me are those of Burnett (1853), Tyson (1870 and 1878), M'Kendrick (1888), Turner (1890 *a* and *b*), Sachs (1890), Hertwig (1893), Karling (1939), and Wilson (1944); those of Mark (1879–80), Strasburger (1880), Waldeyer (1888), Rádl (1930), Conklin (1939), and Woodruff (1939) have also been helpful. Among all the existing literature, however, I have not found a sufficiently detailed or accurate study of the origin and development of the various bodies of opinion. What has been written previously on the subject has helped me mainly by leading, directly or indirectly, to original sources. I have not relied and shall not rely in a single instance on a statement by one author as to what another says. A considerable amount of research has been necessary, which has shed new light on some parts of the cell-theory and contradicted certain generally accepted opinions.

In seeking to affect opinion, scientists are usually careful to make their conclusions verifiable with the least possible trouble. It would be a pleasure to be able to say the same of historians of science, but unfortunately it would also be an unpardonable exaggeration. I am creating something of a precedent by giving exact page-references to most of the sources of my information, except when they are contained in such short papers that this is unnecessary. I intend in this way to make it as easy as possible for readers to check the accuracy of what I say and to correct any errors. All verbal quotations given in this series of papers will be rendered in English. The exact references will make it easy to find the originals in every case. In translating from the various languages, I have tried to be as literal as is consistent with the writing of genuine English: no attempt has been made to preserve any foreign

grammatical forms and thus produce the kind of half-translation that is familiar in scientific literature. I have not relied on the translations of others, except where the author's manuscript was translated before publication, or where the original was not available to me for some other reason. In the case of Swammerdam's *Biblia naturae* (1737–8), which was written by the author in Dutch but printed in Latin and Dutch in parallel columns, I have used the Latin version (except where the contrary is stated). When a printed original source has not been available, I have made it clear that the translation used was not my own, and have given a reference to the translation instead of to the original.

The books used in this investigation have been provided by the Radcliffe and Bodleian libraries and those of the Department of Botany at Oxford, of the Royal Society, the Royal Geographical Society, the British Museum, and the British Museum (Natural History).

I hope that readers who may disagree with the conclusions I draw as to the validity or invalidity of the several propositions may, nevertheless, find some value in the historical parts of this series of papers.

The attempt will be made to express all ideas with the utmost clarity and simplicity, so that there will be no mistake about my meaning. Then, if the ideas are wrong, they can easily be corrected.

In composing the papers I have received help from the criticisms of the cell-theory by Whitman (1893), Sedgwick (1894 and 1895), Bourne (1895, 1896 *a*, *b*, and *c*), Awerinzew (1910), Dobell (1911), Baitsell (1940), Weiss (1940), and Ries (1943). I acknowledge the assistance given by friends in the course of informal discussions. In particular I must mention the encouragement given by Prof. A. C. Hardy, F.R.S., which has helped me in a long and rather difficult task, somewhat removed from the main stream of modern cytological advance.

### PROPOSITION I

*Most organisms contain or consist of a large number of microscopical bodies called 'cells', which, in the less differentiated tissues, tend to be polyhedral or nearly spherical.*

### The Discovery of Plant Cells

Strangely enough, the earliest published account of the microscopical structure of plant tissues is concerned with petrified wood. In describing the resemblances of this material to ordinary wood, Hooke refers to the 'conspicuous *pores*'. He continues: 'Next (it resembled wood) in that all the smaller and (if so I may call those which are only to be seen by a good glass) *microscopical* pores of it, appear (both when the substance is cut and polish'd *transversly*, and *parallel* to the pores) perfectly like the *Microscopical* pores of several kinds of *wood*, retaining both the shape, and position of such pores.' The 'conspicuous *pores*' may have been either resin-canals or large vessels. Most of the '*microscopical* pores' were presumably small vessels, though some

of them may have been cells of the medullary rays or of the wood parenchyma. Hooke regarded them as tubular. He contributed his account of the structure of petrified wood to Evelyn's *Sylva* (1664, p. 96).

Evelyn spells Hooke's name 'Hook', but there is no doubt about the identity of the famous microscopist, for Hooke refers to his study of petrified wood again in his *Micrographia* (1665, p. 100). In this work, before describing cork, Hooke writes '*Of* Charcoal, *or burnt* Vegetables.' In describing the stems of plants, burnt to charcoal and broken across, he first notices the large vessels and proceeds (p. 101): 'But this is not all, for besides those many great and conspicuous irregular spots or pores, if a better *Microscope* be made use of, there will appear an infinite company of exceedingly small, and very regular pores, so thick and so orderly set, and so close to one another, that they leave very little room or space between them to be fill'd with a solid body, for the apparent *interstitia*, or separating sides of these pores seem so thin in some places, that the texture of a Honey-comb cannot be more porous. Though this be not every where so, the intercurrent partitions in some places being very much thicker in proportion to the holes.

'Most of these small pores seem'd to be pretty round, and were rang'd in rows that radiated from the pith to the bark.' He considered the 'pores' to be longitudinal tubes. He calculated that there are 2,700 of them to an inch (in a transverse section). He mentions that in sound wood the microscopical pores 'are fill'd with the natural or innate juices of those Vegetables' (p. 108).

After dealing with charcoal and repeating his already-published observations on petrified wood, Hooke comes to his well-known study '*Of the* Schematisme *or* Texture *of* Cork'. He appears to have seen cells in cork before he studied petrified wood and charcoal, for he writes (pp. 112–15) that the cells of cork 'were indeed the first *microscopical* pores I ever saw, and perhaps, that were ever seen, for I had not met with any Writer or Person, that had made any mention of them before this'. He describes how he cut a very thin section of cork and examined it on a black plate with a plano-convex lens. '. . . I could exceeding plainly perceive it to be all perforated and porous, much like a Honey-comb, but that the pores of it were not regular; yet it was not unlike a Honey-comb in these particulars . . . these pores, or cells, were not very deep, but consisted of a great many little Boxes. . . . Nor is this kind of Texture peculiar to Cork onely; for upon examination with my *Microscope*, I have found that the pith of an Elder, or almost any other Tree, the inner pulp or pith of the Cany hollow stalks of several other Vegetables: as of Fennel, Carrets, Daucus, Bur-docks, Teasels, Fearn, some kinds of Reeds, &c. have much such a kind of *Schematisme*, as I have lately shown [in] that of Cork.' Hooke thought that the 'pith' of the shaft of a feather had a similar structure.

Unlike some of his followers, Hooke did not concentrate the whole of his attention upon the cell-wall. He wrote (p. 116): '. . . in several of those Vegetables, whilst green, I have with my *Microscope*, plainly enough dis-

cover'd these Cells or Poles [misprint for Pores] filled with juices . . . as I have also observed in green Wood all those long *Microscopical* pores which appear in Charcoal perfectly empty of any thing but Air.' This passage provides the earliest mention of the substance of cells (as apart from that of cell-walls), though Hooke naturally did not distinguish between cell-substance and sap.

Grew made a study of the microscopical structure of plants independently of Hooke, and saw the cells. He had finished the 'composure' of his little book (Grew, 1672) when Hooke communicated his observations to him. Grew had supposed that the pith of plants consisted partly of cells like those of a honeycomb and partly of long tubes. Hooke now corrected him on this matter and the correction was accepted (p. 78). Even in this little book, Grew carried the study of plant cells much farther than Hooke. He showed (pp. 78–9) the cellular nature of the cortex as well as the pith and illustrated his findings by a figure (his Fig. 15) of the cortex of the stem of the burdock (*Arctium*) in section. This is probably the earliest-published figure of cells as they occur in a living plant (for Hooke's figures were of petrified wood, charcoal, and cork). Grew carried his researches into a still more important field when he demonstrated the cellular nature of plant embryos. He describes the 'sameness' of the nature of the pith and cortex 'with the *Parenchyma* of the Seed. For, upon farther enquiry with better Glasses, I find, that the *Parenchyma* of the *Plume* and *Radicle*, and even of the *Lobes* themselves, though not so apparently, is nothing else but a Mass of Bubbles' (p. 79).

Ten years later, Grew (1682) published his great book on *The Anatomy of Plants*. The figures, many of them representing microscopical dissections in three dimensions like a modern stereogram, are magnificent. In this work he uses the words 'Bladders', 'Cells', and 'Pores' indiscriminately (see, for example, p. 64). He had now acquired, however, a wrong idea of the units of plant structure. His 'Mass of Bubbles' was a much better analogy than that of '*fine Bone-Lace*' (p. 121), which he develops in a celebrated passage in his later work. This subject will be reviewed under the heading of the Proposition II in the second part of this series of papers.

Meanwhile, Malpighi had been busy with the same subject. At the end of his work, *Anatomes plantarum idea*, he wrote the date 1671. In this paper, which is not illustrated, Malpighi calls cells 'utriculi' and 'sacculi'. The *Idea* was first published four years after it was written, in the large volume, *Anatome plantarum* (Malpighi, 1675). This book included more detailed studies of microscopical plant anatomy. The cellular structure of plants is illustrated by figures of transverse and longitudinal sections of stems. These figures do not approach those of Grew (1682) in elaboration of detail. There has been some discussion as to whether Grew or Malpighi had priority in the microscopical study of plant tissues, and whether Grew was more indebted to Malpighi or vice versa. Schleiden (1849, p. 37) was in error when he said that Malpighi despatched his *Idea* to the Royal Society in 1670. He did not do so until November of the next year. Meanwhile, on 1 May 1671

the Royal Society had already given the order for the printing of Grew's little book (1672). Pollender (1868), who has carefully studied the question of priority, awards it unhesitatingly to Grew (p. 7), while allowing that Malpighi's early work was done quite independently.

Leeuwenhoek was not slow to follow up the work of these early investigators of plant cells, to whom he refers, in an undated letter to Hooke, as 'acutissimos Viros *Malpighium* & *Nehemiam Grew*' (see Leeuwenhoek, 1722, p. 13). Leeuwenhoek here gives a large drawing of part of a transverse section through a stem of oak. His writings on plant cells, however, will not bear comparison with those of Grew, and scarcely with those of Malpighi. On this subject he exhibits to the full his character of dilettante of genius. He repeatedly describes and figures the cells of plants, and continues to do so up till near the end of his life. In a letter written in 1713, for instance, he figures them in the seeds of various plants (see Leeuwenhoek, 1719, pp. 25–6). From his time onwards it was a matter of common knowledge among botanists that plants were constructed largely of microscopical chambers, though vessels were not known to be of cellular origin until much later. (The cellular origin of vessels will be discussed under the heading of Proposition IV, in a later paper in this series.) The following statement by Moldenhawer (1812, p. 86) is representative of the best opinion of his time: '. . . the cellular substance thus consists of single, closed, spherical, oval or more or less oblong, almost cylindrical utricles, which, owing to mutual pressure on one another, assume an angular and flattened form, either regular or more or less irregular, and resembling the cells of a honeycomb.'

## The Discovery of Blood Corpuscles

The absence of thick cell-walls in most animal tissues put zoologists at a great disadvantage, in comparison with botanists, in recognizing the cellular nature of organisms. Complete separateness of cells was the next best help to microscopists after the presence of easily visible cell-walls. The blood is the only part of an animal that can be compared with most plant tissues in the ease with which it reveals its cellular nature.

It will probably never be known with certainty by whom blood corpuscles were discovered. They were certainly seen by Swammerdam, who died in 1680. It is well known that his *Biblia naturae* was published for the first time long after his death. In this great work (Swammerdam, 1737–8, vol. 1, p. 69), he first mentions blood corpuscles in connexion with his description of the dissection of the louse (*Pediculus*): 'If we begin the dissection in the upper part of the abdomen, and cautiously split the skin there, blood immediately escapes from that place. The blood, when received into a glass tube and examined with a very good microscope, is observed to consist of transparent globules (globulis), in no way differing from cow's milk, a fact that was discovered a few years ago in human blood also; for this is seen to consist of slightly reddish globules, floating in a clear fluid.' It will be noticed that Swammerdam does not state that it was he who made the discovery in human

blood. He remarks: '. . . I shall not recklessly assert that globules are present in the blood of the louse, for it could easily happen that fat might mix itself [with the blood], and so also might certain fragments of the viscera, damaged [by the dissection]; these certainly consist of a mass, as it were, of globular particles, as I shall show at the proper time.' He illustrates the globules, as seen within the glass tube, in Fig. 1 of Tab. II. He did not realize that they might be those of human blood sucked by the louse.

Swammerdam refers to the blood corpuscles of the frog in the second volume of the same book. He writes (p. 835): 'In the blood I saw a watery part, in which floated an immense number of circular particles, rejoicing in a flat, as it were oval, but perfectly regular shape. These particles seemed also to contain another fluid again within themselves. But if I looked at them from the side, they resembled crystalline rods and many other figures; according, doubtless, to the various ways in which they were rotated in the fluid of the blood. I observed moreover that the larger the objects were represented through the intervention of the microscope, the paler their colour appeared [to be].'

Flloyd's translation of the *Biblia* (Swammerdam, 1758) suggests strongly, on p. 120, that Malpighi and Needham already knew of the presence of globules in blood before Swammerdam. The original work was produced in Dutch and Latin in parallel columns. In the passage which Flloyd is here translating, neither the Dutch nor the Latin version gives any evidence for the suggestion that Malpighi or Needham had priority over Swammerdam in this matter.

Unfortunately there would seem to be no means of dating these observations. Foster (1901, p. 99) says that Swammerdam discovered blood corpuscles in 1658 but gives no evidence for this. Miall (1912, p. 198) called attention to the lack of evidence, and no answer appears ever to have been made. It is quite possible that Swammerdam demonstrated the blood corpuscles to the Duke of Tuscany, when the latter visited him and offered him employment. If a record of this particular demonstration exists, the attention of present-day biologists should be called to it. Swammerdam himself (1737–8, p. 839) mentions the Duke's visit, and tells how he demonstrated a nerve-muscle preparation on this occasion. Swammerdam gives 1658 as the date of the visit, but Boerhaave, in writing the great naturalist's biography as a preface to the *Biblia*, states that the date was 1668 (page facing p. C2). Stirling (1902, p. 23), like Foster, gives 1658 as the date of Swammerdam's discovery of blood corpuscles but provides no new evidence.

In his *Exercitatio de omento, pinguedine, et adiposis ductibus*, published in 1665, Malpighi makes the first mention in print of blood corpuscles (see Malpighi, 1686, vol. 2, p. 41). It cannot be claimed that the discovery was made in a satisfactory manner, for he regarded them as globules of fat; yet it is clear from his words that he saw them and that he had no previous knowledge of the existence of such objects in blood. He wrote: '. . . in the omentum of a hedgehog, in a blood-vessel that extended from an accumulation of

fat to another opposite to it, I saw globules of fat, possessing an outline of a particular shape, and reddish; they resembled in general a circle (coronam) of red corals.'

In a letter written to the Royal Society in 1673 (or possibly in 1674), Leeuwenhoek thus describes his own discovery of blood corpuscles (Leewenhoeck [*sic*], 1674, p. 23): 'I have divers times endeavoured to see and to know, what parts the *Blood* consists of; and at length I have observ'd taking some Blood out of my own hand, that it consists of small round globuls driven through a Crystalline humidity or water: Yet, whether all Blood be such, I doubt. And exhibiting my Blood to my self in very small parcels, the globuls, yielded very little colour.' Four years later, Hooke (1678, p. 93) attributed the discovery of the 'Globules' of the blood to Leeuwenhoek.

In his characteristically random manner, Leeuwenhoek reverted to blood corpuscles from time to time. In a letter written to the President of the Royal Society in 1683 (three years after Swammerdam's death), he described and figured the red corpuscles of the frog, noting carefully how their colour appears more clearly when two or three are superimposed (see Leeuwenhoek, 1722, pp. 54–5). Writing to the Royal Society again in July 1700, he makes the first mention of the nucleus ('lumen'), and figures it, in describing the blood corpuscles of the salmon and flounder (see Leeuwenhoek, 1719, pp. 219–20). (The discovery of the nucleus will be more fully considered in the discussion of Proposition II.) Writing yet once again to the Royal Society in his old age, in 1717, Leeuwenhoek gives the first indication that human red blood corpuscles are not spheres, but concave disks (Leeuwenhoek, 1719, pp. 421–2). Sixty years later, Hewson (1777, p. 15) observed that mammalian blood corpuscles were not spherical, as was still commonly supposed, but flat, and therefore, he concluded, not fluid.

Although blood corpuscles were discovered at about the same time as the cells of plants, and both soon became familiar objects, it did not occur to the early microscopists that there was any relation between them. It was necessary first to understand that the non-fluid tissues of animals also consisted of immense numbers of minute microscopical bodies. This understanding came, in a roundabout way, through the globule-theory. But before we can follow the tortuous course of progress, it is necessary to clear away a fallacy that has misguided historians of science and given a false impression of the background of the cell-theory.

## The 'tissu cellulaire' Fallacy

The expression 'tissu cellulaire', or its counterpart in other languages, occurs frequently in eighteenth- and early nineteenth-century writings on the tissues of animals. Alighting casually upon this phrase, one may easily fall into the error of supposing that it refers to cellular tissue, in the modern sense of the word 'cell'. Historians of the cell-theory, among them Gerould (1922), have been misled by this fallacy. Biologists owe a particular debt to Turner (1890a) and Wilson (1944) for calling attention to this matter.

Haller (1757) devotes Sectio II of his *Elementa physiologiæ corporis humani* to what he calls cellular tissue ('Tela cellulosa'). One has only to read what he writes on this subject (p. 9) to realize that he is using the phrase to mean what we should call areolar connective tissue. This appears even more evidently from his 'First lines of physiology' (Haller, 1779). The translator from the Latin of the 1766 edition renders the relevant passage as follows (pp. 3–8):

'The second kind of fibres . . . when loosely interwoven with each other, are called the *cellular* tunic; though the name *tunic* or *membrane* is on many accounts very improper.

'This cellular substance is made up of an infinite number of little plates or scales, which, by their various directions, intercept small cells and web-like spaces; and join together all parts of the human body in such a manner, as not only sustains, but allows them a free and ample motion at the same time. But in this web-like substance there is the greatest diversity, in respect of the proportion betwixt the solid parts and intercepted cells, as well as the breadth and strength of the little plates, and the nature of the contained liquor, which is sometimes more watery, and sometimes more oily. . . . This cellular web-like substance in the human body is found throughout the whole, namely, wherever any vessel or moving muscular fibre can be traced; and this without the least exception that I know of. . . . The principal use of the cellular fabric is to bind together the contiguous membranes, vessels, and fibres, in such a manner as to allow them a due or limited motion. . . . The intervals or spaces betwixt the plates or scales of the cellular membrane, are every where open, and agree in forming one continuous cavity throughout the whole body.'

It is clear that the 'cells' referred to in this passage have nothing to do with cells in the modern sense: they are simply the areolae of connective tissue.

Gerould (1922), who attributes the cell-theory to Lamarck (1809), has been misled by the words 'tissu cellulaire'. There are, indeed, some passages in Lamarck's *Philosophie zoologique* from which one might conclude that he was referring to cellular tissue in the modern sense. For instance, he says 'that the whole operation of nature for the formation of her direct creations, consists in organizing *"en tissu cellulaire"* the little masses of gelatinous or mucilaginous matter that she finds at her disposal and favourable in the circumstances' (1809, vol. 1, p. 373). A careful study of his work leaves no doubt, however, that when he refers to 'tissu cellulaire' in animal tissues, he is never referring to cells in the modern sense, but is nearly always referring to connective tissue. There are a few exceptions. In the case of polyps (vol. 1, p. 203), he appears to use the expression to mean mesogloea, while when he says (vol. 1, p. 210) that infusoria are composed of 'tissu cellulaire', we cannot guess his precise meaning. His words on p. 46 of vol. 2, however, leave no doubt whatever of the usual sense. They must be quoted in full as they settle the matter conclusively:

'. . . all the organs in animals without exception are enveloped in *tissu cellulaire*, and their lesser parts are in the same case.

'In fact, it has been recognized for a long time that the membranes that form the envelopes of the brain, of the nerves, of the vessels of all kinds, of the glands, of the viscera, of the muscles and their fibres, even the skin of the body are generally productions of *tissu cellulaire*.'

Another passage clearly indicating that 'tissu cellulaire' means connective tissue occurs on p. xiv of vol. 1. His repeated insistence (vol. 1, pp. 273 and 409; vol. 2, pp. 47 and 60) that 'tissu cellulaire' is the 'gangue' in which structure is laid down is another pointer in the same direction; for 'gangue' is the substance that encloses a metallic ore in its meshes, not the ore itself. Lamarck's ideas on morphogenesis are so unfamiliar to-day that it is difficult at first to grasp them. He makes himself clearest on this subject on pp. 373–4 of vol. 1. He regarded connective tissue as playing a fundamental part in the development of structure. Fluids move through the meshes of this tissue, and the effect of this movement is 'to open up (frayer) routes . . . to create in it canals, and consequently various organs; to vary these canals and their organs by reason of the diversity either of the movements or of the nature of the fluids'.

When dealing with plants, Lamarck uses the expression 'tissu cellulaire' to mean the cell-walls, or sometimes, more loosely, to mean the cell-walls and their contained fluids. He is therefore thinking of cells in something approaching the modern sense. It is important to realize that he homologized the cell-walls of plants with the connective tissue fibres of animals. This supposed homology seems to us so extraordinary that we do not readily understand his meaning.

Bichat (1812) devotes no less than 104 pages to the 'Système cellulaire'. What he writes at the outset (p. 11) makes it perfectly clear that by this expression he means areolar connective tissue, with the cells of which, in our modern sense, he is not at all concerned. So persistent was the term 'Zellgewebe', that Schwann himself uses it (1838*b*, col. 227) in the old sense when he wants to refer to areolar connective tissue, though in the same breath he mentions the true nucleated cells contained in it. The term 'connective tissue' is so familiar to ourselves that we may perhaps omit to reflect that it required to be invented and only gradually displaced a misleading but very familiar expression. Possibly the first use of it, in the form of 'Bindegewebe', occurs on p. 444 of Müller's *Handbuch der Physiologie* (1834), where he is discussing the histology of the kidneys, liver, &c.

*The Globule-theory*

What may be called the globule-theory was to some extent the forerunner of the cell-theory. Here again historians have been misled. Casual reading has suggested that various early authors knew much more about cells than in fact they did. Yet some of the 'globules' were actually cells, and to that extent the globulists were on the path of progress. The historian's difficulty is to disentangle the occasions on which they saw cells from those on which they did not.

It has already been mentioned that Swammerdam (1737–8, p. 70), in his study of the louse, stated that its viscera 'certainly consist of a mass, as it were, of globular particles (partium globulosarum), as I shall show at the proper time'. He says (p. 76) that the coats of the stomach, especially the external one, consist 'of very numerous . . . globular granules (granulis globosis)', which he describes as 'somewhat irregular'. He cannot decide whether these granules are part of the texture of the stomach or fat-particles. He remarks also (p. 70) that the muscles of the louse, when dried on glass and washed with spirits of wine, appear distinctly to be composed of globules, and also (p. 81) that the membrane that covers its nerve-ganglia seems to be composed of irregular globular particles (globosis particulis). He gives rather a confused description (pp. 84–5) of the structure of the skin, remarking that the smallest change of focus produced a new appearance. He sometimes saw 'globosae particulae' in it; sometimes it appeared to be composed of regular squares, which are illustrated in his Fig. x of Tab. II.

It is not possible to decide which of these various globules, if any, were cells and which were not. It would seem probable that he saw cells in the coats of the stomach, while the globules in the muscles may perhaps have been nuclei. Unfortunately these observations cannot be exactly dated. Boerhaave tells us in his Preface to the *Biblia* (on the page opposite p. F.2) that Swammerdam did no more scientific work after he had finished his history of the mayfly in 1675; it will be recollected that he died in 1680.

Miall (1911, p. 103), referring to the *Biblia*, says that Swammerdam 'describes a stage in which the body [of the tadpole] is entirely composed of rounded "lumps" or "granules", the *cells* of modern biology'. He repeats this on p. 106. Miall is here mistaken. On page 817 of the *Biblia* (vol. 2), Swammerdam does indeed give the impression that he knew the tadpole to be composed of cells; but it is clear from what he says at the end of the paragraph that on this occasion he is only referring to yolk-grains when he writes of 'globosas particulas'.

The year 1665 saw the first descriptions in print of what are nowadays regarded as cells of animals by Malpighi and Hooke. The former, in his *Exercitatio de omento*, refers to his microscopical examination of 'Pinguedinis globuli' and 'adiposi globuli' (reprinted in his *Opera omnia*, 1686, vol. 2, see p. 4). There would not appear to be any doubt that these were fat-cells, though it was presumably the fat-globules themselves that struck his attention, rather than the cells that contained them. Hooke (1665, p. 158) describes the hair of an Indian deer as seen under the microscope. In his figure (F in Fig. 3 of Schem. V), the imbricating scales of the cuticle of the hair are clearly seen. It looks, he says, 'like a thread of course Canvass, that has been newly unwreath'd, it being all wav'd or bended to and fro, much after that manner'. He only saw the externally projecting parts of the cuticular scales, not the complete transformed cells.

Leeuwenhoek also observed the structure of hair. He says that he examined his own hair, 'which heretofore I imagined to have seen to grow out of globuls

. . . so that Hair grows and increases by the protrusion of globuls. But two or three days agoe I observed the Hair of an *Elk*, and found it wholly to consist out of conjoyned globuls, which by my Microscope appear'd so manifestly to me, as if they could be handled' (Leewenhoeck [*sic*], 1674, pp. 23–4). Just what these globules were is uncertain; presumably they were the imbricating scales as they appeared with diffraction haloes round them.

Leeuwenhoek was now launched on his globule-finding studies, throughout which there is a curious mixture of truth and error. In the same paper he notices the 'small transparent globuls' of cow's milk, but also finds, inexplicably, that his nail consists of globules, and has no doubt that it grows from 'globuls protruded'. The most misleading of his researches in this line led to his report of the existence of globules in the brain (1686, pp. 883–9). In that of a turkey he observed 'some extream small Globules, less than 1/36th part of one of those which make the rednes in the blood'. There were also some about one-sixth of the size of a red blood corpuscle. He thought that these might have come from blood-vessels broken by himself. 'Together with the above mentioned Globules, there were some transparent irregular ones, as big or bigger than a Globule of our blood, which lay among the branches of the blood Vessels, in a space no bigger than a coarse sand.' He also found globules of various sizes in the medullary parts of the same brain, and in the brains of a sheep, ox, and sparrow.

There is nothing in this paper that could convince anyone that Leeuwenhoek saw the cells of nervous tissue. He refers more than once to his animals having been dead a considerable time before examination, and mentions (p. 885) that some of the globules seemed to consist 'of a thin transparent Oyl-like substance'. It is probable that Leeuwenhoek was looking at lipoid particles derived by maceration from cells and fibres. His description of globules in the brain, however, had a profound influence on subsequent writers.

In a letter written in 1717, Leeuwenhoek describes a transverse section of a small nerve, and gives a figure in which the fat-cells lying between the bundles of nerve-fibres are clearly seen (Leeuwenhoek, 1719, Fig. 2 on the plate opposite p. 312). He calls the fat-cells 'partes adiposae'. Monro (1726, p. 21) is probably referring to fat-cells when he describes the marrow of human bone. '. . . the Marrow,' he says, 'when hardened and viewed with a Microscope, appears like a Cluster of small Pearls. When the Oil is evaporated, these Bladders seem exactly like the *Vesiculae* of the Lungs when blown up, but not so large. The Marrow is nothing but the more oily Part of the Blood separated by the small Arteries, and deposited into these *Cellulae*.'

The foregoing observations are disconnected. With Wolff, the epigeneticist, we come to a generalization, referring to the minute structure of the embryos of animals. 'The constituent parts,' he says, 'of which all parts of the animal body are composed at their first beginnings, are globules (globuli), which always yield to a moderately good microscope' (Wolff, 1759, p. 72). He gives a figure (Fig. 1 on Tab. II) of a 28-hour chick embryo; cells can be

seen in the area pellucida. (This figure is poorly reproduced in the second edition of the book (Wolff, 1774).)

Hewson (1777, pp. 63–81) investigated the microscopical structure of various glands of the lymphatic system. 'On cutting into a fresh lymphatic gland', he says, 'we find it contains a thickish, white, milky fluid. Then if we carefully wipe, or wash this fluid from any part of it, and examine it attentively in the microscope, we observe an almost infinite number of small cells, not such as have been before described, or that have been supposed to exist in the lymphatic glands, but others too small to become visible to the naked eye, expressed Plate IV. Fig. 4.' He dilutes the fluid with a solution of Glauber's salt or with blood-serum and then sees 'NUMBERLESS small, white, solid particles, resembling in size and shape those central particles found in the vesicles of the blood'. Hewson found similar 'particles' in the thymus gland and spleen. Although both his description and his figures suggest too small a size for these particles, in relation to red blood corpuscles, yet there would not seem to be much doubt that the objects of his study were lymphocytes and thymocytes. It is curious that he calls them 'cells'. He does not suggest any relation to the cells of plants.

Leeuwenhoek's ideas on the globular structure of nervous tissue had an unfortunate influence on several workers towards the end of the eighteenth and beginning of the nineteenth century. Prochaska (1779, pp. 67–8) wrote as follows: 'For when a small piece of the substance of either the cortical or the medullary part of the cerebrum or cerebellum is placed on a very thin glass and flattened out, so that it becomes conveniently transparent, then, with the help of a selected optical lens, it is revealed to be as it were a thick paste consisting of innumerable globules (globulis), in which no movement or swimming can be observed.' Prochaska tried by dilution and prolonged maceration to separate the globules completely from one another, but without success; and he concluded that they must be held together by a very subtle and very transparent connective tissue ('tela cellulari'). He describes the shape of the globules as 'not exactly spherical but irregularly rotund' (p. 70). He figures them on Figs. VIII–XI on Plate VII.

Reference has been made by a number of historians to Prochaska's work, but it does not seem to have been carefully read by them. It is certain that Prochaska did not see nerve-cells; for his globules were less than one-eighth the size of red blood corpuscles (p. 72), and they occurred in the nerves (pp. 70, 73) as well as in the brain. Despite this, it has even been said that Prochaska saw the nuclei of nerve-cells. His Fig. X does suggest this at a glance; but he describes carefully the various appearances obtained as he focused his lens on the globules, and there can be no doubt that the figure shows merely the effect of spherical aberration. This error, together with haloes produced by lenses of small numerical aperture, must often have led the globulists astray.

Fontana (1781, p. 212) also describes 'petits corpuscules ronds' in both the cortical and medullary parts of the brain. He figures them in Fig. VI of

Plate V, but it is not possible to identify them. He differed from Prochaska, however, in his account of the structure of nerves (p. 234): here he found 'cylindres tortueux primitives', which he considered to be of an elementary nature ('des principes simples primitifs, non composés d'autres moindres'). He regarded such primitive cylinders as being the basis also of tendons and muscles. He describes globules in the 'gluten' of the skin of eels. These globules, which appear to have been cells, will be described in the discussion of Proposition II. He also found globules in the retina of the eye. (Fontana repeatedly uses the phrase 'tissu cellulaire' to mean areolar connective tissue.)

Oken must be regarded as a globulist, but there is a characteristic absence of any objective descriptions that would enable his readers to check the truth of what he says. In his remarkable work, *Die Zeugung* (1805), he develops his thesis that higher organisms are constructed from a mass of infusoria. This matter will be considered further in its proper place, in the discussion of Proposition VI. A few years later (Oken, 1809, p. 26) he makes some dogmatic general remarks on the structure of organisms:

'A sphere, of which the middle is fluid but the periphery solid, is called a *bladder* (*Blase*).

'The first organic points are little bladders (Bläschen). The organic world has for its basis an infinity of little bladders.'

Many years afterwards, when the scientific world was resounding with the fame of Schwann, Oken claimed the cell-theory for himself: 'I first instituted my doctrine that all organisms arise from and consist of *little bladders* or *cells* in my book on Reproduction (Frankfurt bei Wesche, 1805, 8vo.). These little bladders, isolated and considered in their primitive origin, are the infusorial substance or primitive slime, from which all larger organisms form themselves. . . . This doctrine of the primitive constituents of the organic substance is now generally acknowledged, and I need add nothing, therefore, to the advocacy of it' (Oken, 1843, p. iii). With a characteristic stroke of genius, Oken seems to have glimpsed the homology of the cells of plants with the globules that were coming to be recognized as frequent components of animal tissues; but his writings were not very influential, because no exact observations were recorded.

Globulism reached its zenith in the brothers Josephus and Carolus Wenzel. They were clearly influenced by Prochaska, whom they quote at length. They found 'globuli', 'cellulae', or 'corpuscula' in the brains of man, rabbit, sheep, duck, fowl, pigeon, redpoll, and carp (1812, pp. 27–36). They describe them as sub-rotund, but sometimes somewhat angular. Any tendency to suppose that they actually saw nerve-cells is opposed by their finding that nerves also are composed of similar corpuscles. They found spherical corpuscles, however, in various other tissues, and some of these may have been cells. Their general conclusions are so sweeping, and seem to forestall the cell-theory in such a surprising manner, that they deserve to be quoted; but the reader should remember the weakness of the evidence on which their generalizations are based. They conclude that:

'The cortical and also the medullary substance of the human cerebrum and also of the cerebellum;

'The substance of the colliculi that are found in the interior of the human cerebrum;

'The substance of the pineal gland, of the spinal medulla, and of the nerves;

'Finally, the mass of the cerebrum in mammals, birds and fishes, consist of the same small, mutually coherent, sub-rotund corpuscles, of which the substance of muscle, liver, spleen, and kidneys is composed.'

Their final conclusion is that 'the particular structure of the whole of the cerebrum and nerves and also of all the other organs is cellular (cellulosam)....Finally, that the principle (Principium), or fundamental structure, of all the solid parts without exception is one and the same.'

Meckel (1815) was the first to incorporate the globule-theory in a text-book of anatomy. He regarded organisms as made up of two ultimate constituents: globules (Kügelchen), and 'a *coagulated*, or *coagulable* and therefore *plastic* substance' (1815, p. 4), in which the globules are invariably embedded. The globules are not always exactly spherical, but they are never angular. Apart from the blood, in which they assume particular shapes, all the globules of the body of any one species of animal have the same form; they are never elongated in one part and round in another. In man they are round. They are larger in the spleen than in the kidneys, and in the latter than in the liver. The milk globules are of the same nature as those of other organs.

Treviranus's contribution (1816) to the globular theory was not important. He remarks that all good observers see the globules (Kügelchen) in the brain. He recognizes three main elements in the tissues of animals (p. 140): elementary cylinders, protein globules (Eyweisskügelchen), and formless material. He also recognizes elementary fibres, which appear to have been connective tissue fibres, and which he regarded as of plant-like nature; but he denies (p. 126) that there is any trace in animals of anything resembling the cellular tissue of plants. It is difficult to identify Treviranus's Kügelchen; in some cases they may have been nuclei, in others artifacts, in *Hydra* nematocysts. His figures are unhelpful and show nothing that can be recognized as a cell.

If Treviranus's contribution is confused, Home's (1818, 1821) is simply erroneous. His studies were made in collaboration with a Mr. Bauer, who seems to have done most of the practical work. They noticed (1818) that when blood coagulates, the red blood corpuscles tend to unite in lines. They then boiled or roasted voluntary and involuntary muscle, macerated it in water, and found that the fibres 'are readily broken down into a mass of globules of the size of those in the blood, deprived of their colour' (1818, p. 175). They concluded that muscle is formed by the joining together of red blood corpuscles in lines, and suggested that nerve-fibres were formed in the same way. In a later paper (1821) Home describes both nerves and brain as containing innumerable globules, from 1/2,000 inch (6μ) to 1/4,000 inch in diameter. He evidently considered them to be derived from the red corpuscles of the blood. It is strange that Prevost and Dumas (1821), who

were later to make an important advance in science by the discovery of cleavage, agreed with Home's opinion that muscle fibres are formed by the arrangement of red blood corpuscles in lines.

Heusinger (1822) shows affinity with Home, and indeed carries his ideas farther. He considered (pp. 113–16) that the tissues of the body had three constituents: formless matter, globules (Kugeln), and bladders (Blasen). The first he regarded 'as the mother, as the primitive sea of all other tissues'. Fibres are formed by the arrangement of globules in a row: bladders arise from globules by the development of a differentiated pellicle, and themselves give rise to vessels by arrangement in rows and confluence of their cavities. Heusinger generalizes freely and gives little precise information: his style is reminiscent of Naturphilosophie.

Milne Edwards (1823) was strongly influenced by Prochaska, Fontana, and Wenzel. He made a systematic study of the microscopical structure of many organs. He found 'globules' in the connective tissue of man and various animals, in the peritoneum, conjunctiva, the mucous membrane of the intestine, voluntary muscle, tendon, skin, the walls of arteries and veins, and in the white and grey matter of the brain. In nearly every case he notes that the globules are 1/300 mm. in diameter. Unfortunately he gives no figures, and it is impossible to guess exactly what he saw. In some cases he may have been looking at nuclei, in others at lipoidal droplets, in others again he may have seen cells; but if he did, it is difficult to account for their uniformly spherical shape and minute and unvarying size.

The last in the direct succession of the globulists was Dutrochet (1824). Himself mainly a botanist, he relied to a large extent on Milne Edwards for his information about the microscopical structure of animals; but he claims (p. 201) to have verified the latter's observations. For him, 'all the organs of animals are composed of agglomerated globular corpuscles' (p. 13; see also pp. 200–1).

Although there was some truth in the claims of the globulists, and although they did pave the way for a true understanding of the microscopical structure of animals, yet some check to their errors was urgently needed. It was provided by Hodgkin and Lister (1827). Using the improved microscope designed by Lister, they found no globules, but only fibres, in striated muscle and in the muscle of arteries. They looked in vain for globules in nerves. They saw no globules in brain, but only very small particles, which they regarded as resulting from the disintegration of the tissue. They saw no globules in connective tissue (or 'cellular membrane', as they called it). They found human red blood corpuscles to be concave, and the particles of pus to be irregular in shape. They found globules only in milk. They were aware that their results differed from those of Milne Edwards, who was a friend of Hodgkin, and attributed the difference to the imperfection of Edwards's microscope. There can, indeed, be little doubt that many of the globules reported by the early microscopists were images of minute particles, smaller than any ordinary cells, but surrounded by haloes. The fact that the

excesses of the globulists were exposed by Lister's microscope seems significant; for the particular advantage of his instrument was that spherical aberration was corrected and the 'ring' appearance round small particles thus reduced. His objectives, though not perfected by this time, must already have been good. The work of Hodgkin and Lister was a healthy and much-needed corrective. They were supported by Grainger (1829), whose own observations agreed with theirs.

The time had now arrived when microscopists were beginning to see actual cells in various animal tissues. Von Baer (1828, pp. 144–5) noticed that the elements of which an embryo consists—fibres, globules, and platelets—become smaller as development proceeds. He uses the words Kügelchen and Körnchen interchangeably. He says of the Körnchen in the developing chick that they 'are so large, in relation to the parts that they compose, that one might say that the embryo at a very early stage resembles a picture made of paving-stones or blocks of granite. On the first day the notochord consists almost entirely of one row of such globules (Kügelchen), which one can count with tolerable accuracy . . . the individual globules can be distinguished in the embryonic area with moderate magnification; the embryo appears to contain several hundreds of them.' There is no reason to doubt that these Körnchen and Kügelchen were cells.

Dutrochet (1837) illustrated a small fragment of the brain of the frog as seen under the microscope. The figure (Fig. 3 on Plate 30) shows a large number of cells and a small vessel running among them. They were probably nerve-cells, though the figure does not permit this to be concluded with certainty. It must be allowed that Dutrochet was not an exponent of animal histology. He remarks (p. 470) that 'when observing with the microscope the tissue of the brain, the liver, the kidneys, the spleen, etc., in a frog, for instance, one really notices no difference'. Purkinje (1838) found that the most diverse organs consist of Körner, Körnchen, or Cylinderchen, often associated with fibres. He found this to be true of the glands of the mammalian stomach, the liver, the salivary glands, the pancreas, unspecified mucous glands, the ear-wax glands, kidneys, testes, epididymides, epidermis, mucous epithelia, ciliated membranes of the respiratory tract and of the female genital system, spleen, thymus, thyroid, and lymph-glands. His paper has a more modern aspect than those of the globulists, and there is reason to believe that he saw the cells of most or all of these organs. The Cylinderchen were the cells of columnar epithelium. By this time, however, the nucleus was beginning to be identified in various tissues. This provided a criterion by which a cell could be recognized as such. The subject now falls within the scope of Proposition II, and will be discussed further under that heading in the second part of this series of papers.

## Early Comparisons of Plant and Animal Cells

When considering plant tissues, most of the early observers concentrated their attention upon the cell-walls, which they thought to form a continuous

meshwork. Either this meshwork corresponded to the fibres of connective tissue, as Lamarck and the others supposed, or else it seemed to have no counterpart at all in the animal kingdom, in which the tissues consisted largely of 'globules'. Microscopists were slow to realize that the utricles held in the meshes of plant cell-walls might correspond to the globules.

The purpose of this section of the paper is to give some early examples of cases in which actual cells of plants and animals were compared.

Oken's generalizations on this subject have already been quoted (p. 118). It is impossible to be sure that he saw the cells of animals.

Dutrochet (1824, pp. 14–15) said that there is a 'similitude évidente' between the microscopical structure of the brain of gastropods on the one hand and of the pith of *Mimosa pudica* on the other. Raspail (1833, pp. 187, 191) compared the microscopical structure of fat with that of plant tissue. He speaks of 'the analogy of this animal cellular tissue with vegetable cellular tissue'. Valentin (1835, p. 287) described the mesoderm (Gefässblatt) of the chick embryo as composed of large Kugeln, so tightly crowded together 'that they are flattened at many points of contact and often, as [in] the cellular tissue of plants, assume a hexagonal form'. Valentin (pp. 209–10) also refers to a condition resembling the cellular tissue of plants in the ossifying cartilage of the labyrinth of the ear, but it is doubtful whether he is here referring to cells. Müller (1835, p. 25) wrote as follows of the notochord of *Myxine glutinosa*, as seen in transverse section under the microscope: 'The cells are irregular, and unlike one another, but resemble the cells of plants to some extent in that the walls seem to be closed on all sides and mostly touch one another in straight lines, so that irregularly polygonal figures appear in transverse sections.' Müller shows this in Fig. 1 of Plate IX.

Turpin (1837) was led to compare the cells of plants and animals when he undertook a critique of the microscopical studies of a certain Dr. Donné on the liquids secreted and excreted by organic tissues. To check the accuracy of Donné's statements, Turpin repeated most of the observations. Donné had described what were evidently squamous epithelial cells of the human vagina. Turpin says (p. 210): 'After having thoroughly studied the vesicles forming the layer of mucus produced by the vaginal mucous membrane, one cannot avoid seeing in it a cellular tissue that is well-organized and composed, like all vegetable cellular tissues, of an agglomeration by simple contiguity of distinct vesicles living *individually* each on its own account at the expense of the mucous fluid that bathes them on all sides. This animal cellular tissue . . . may be rigorously compared with that of many vegetable cellular tissues.'

Dutrochet reverted to the comparison of plant and animal cells many years after making his first contribution to this subject. He made a direct comparison between the cells of plants and those of the salivary gland of *Helix* (1837, pp. 469–70). 'One sees from that', he remarks, 'that nature possesses a uniform plan for the intimate structure of organised beings, both animal and vegetable.'

As a result of the extensive histological researches that have already been mentioned, Purkinje (1838, p. 175) also drew a comparison of plant and animal cells. 'Consequently,' he wrote, 'the animal organism almost completely reduces itself to three main elementary forms: the fluid, the granular (körnige), and the fibrous. The granular ground-form suggests again an analogy with the plant, which, as is well known, is almost entirely composed of granules or cells (Körnern oder Zellen).'

*Comment*

The following is the essence of the ideas briefly summarized in the first proposition:

The tissues of most organisms, when examined under the microscope, are seen not to be perfectly continuous, for there is generally a partitioning by cell-membranes, cell-walls, and intercellular matter of various kinds; and this partitioning leaves much of the material of the organism in the form of more or less separate bodies to which the name *cells* is applied. These cells are usually of relatively simple shape in the less differentiated tissues (spheroids, simple polyhedra, &c.).

The facts recorded in this first paper are mainly of historical interest, for the truth of the first proposition is generally admitted and little in the way of critique is possible. It must be remarked, however, that although the knowledge summarized in the proposition was fundamental for the establishment of the cell-theory, yet those who got the knowledge were not at the time in a position to envisage the large superstructure that would eventually be built on their foundations. The first necessary advance was the production of evidence that the various objects that were called cells had in fact important characters in common that made it proper to include them all under a single name. That is the subject of the second proposition which will be considered in the second of this series of papers.

## REFERENCES

AWERINZEW, S., 1910. Biol. Centralbl., **30**, 465.

v. BAER, K. E., 1828. *Über Entwickelungsgeschichte der Thiere. Beobachtung und Reflexion.* Erster Theil. Königsberg (Bornträger).

BAITSELL, G. A., 1940. Amer. Nat., **74**, 5.

BICHAT, X., 1812. *Anatomie générale, appliquée à la physiologie et à la médecine.* New edit. Vol. 1. Paris (Brosson, also Gabon).

BOURNE, G. C., 1895. Quart. J. micr. Sci., **38**, 137.

—— 1896a. Sci. Prog., **5**, 94.

—— 1896b. Ibid., **227**.

—— 1896c. Ibid., 304.

BURNETT, W. J., 1853. Trans. Amer. Med. Assoc., **6**, 645.

CONKLIN, E. G., 1939. Amer. Nat., **73**, 538.

DOBELL, C. C., 1911. Arch. Protistenk., **23**, 269.

DUTROCHET, M. H., 1824. *Recherches anatomiques et physiologiques sur la structure intime des animaux et des végétaux, et sur leur motilité.* Paris (Baillière).

—— 1837. *Mémoires pour servir à l'histoire anatomique et physiologique des végétaux et des animaux.* 2 vols. and atlas. Paris (Baillière).

E(VELYN), J., 1664. *Sylva, or a Discourse on Forest-trees, and the Propagation of Timber in His Majesties Dominions.* London (Martyn and Allestry).

FONTANA, F., 1781. *Sur les poisons et sur le corps animal.* 2 vols. Paris (Nyon); London (Emsley).

FOSTER, M., 1901. *Lectures on the History of Physiology during the Sixteenth, Seventeenth and Eighteenth Centuries.* Cambridge (University Press).

FRÉDÉRICQ, L., 1884. *Théodore Schwann: sa vie et ses travaux.* Liége (Desoer).

FULINSKI, B., 1916. Zool. Anz., **47**, 381.

GEROULD, J. H., 1922. Sci. Mon., **14**, 268.

GREW, N., 1672. *The Anatomy of Vegetables begun. With a General Account of Vegetation founded thereon.* London (Hickman).

—— 1682. *The Anatomy of Plants. With an Idea of a Philosophical History of Plants. And Several Other Lectures, read before the Royal Society.* Publ. by the author (place not stated).

HALLER, A., 1757. *Elementa physiologiæ corporis humani.* Lausannæ (Bousquet).

—— 1779. *First Lines of Physiology.* Transl. from the correct Latin edition. Edinburgh (Elliot).

HERTWIG, O., 1893. *Die Zelle und die Gewebe. Grundzüge der allgemeinen Anatomie und Physiologie.* Jena (Fischer).

HEUSINGER, C. F., 1822. *System der Histologie.* Eisenach (Bärecke).

HEWSON, W., 1777. *Experimental Enquiries: Part the Third.* Edit. by M. Falconar. London (Longman).

HODGKIN, —, and LISTER, J. J., 1827. Phil. Mag., **2**, 130.

HOME, E., 1818. Phil. Trans., **108**, 172.

—— 1821. Phil. Trans., **111**, 25.

HOOKE, R., 1665. *Micrographia: or Some Physiological Descriptions of Minute Bodies made by Magnifying Glasses. With Observations and Inquiries thereupon.* London (Martyn & Allestry).

—— 1678. *Lectures and Collections made by Robert Hooke, Secretary of the Royal Society.* London (Martyn).

KARLING, J. S., 1939. Amer. Nat., **73**, 517.

LAMARCK, J.-B.-P.-A., 1809. *Philosophie zoologique, ou exposition des considérations relatives à l'histoire naturelle des animaux.* Paris (Dentu, and from the author).

LEEWENHOECK [sic], 1674. Phil. Trans., **9**, 23.

LEEUWENHOEK, A., 1686. Phil. Trans., **15**, 883.

—— 1719. *Epistolæ physiologicæ super compluribus naturæ arcanis.* (Vol. 2 of the *Opera omnia.*) Delphis (Beman).

—— 1722. *Opera omnia.* Vol. 1 of Editio novissima. Lugduni Batavorum (Langerak).

MALPIGHI, M., 1675. *Anatome plantarum. Cui subjungitur appendix, iteratas & auctas ejusdem authoris de ovo incubato observationes continens.* London (Royal Society).

—— 1686. *Opera omnia, figuris elegantissimis in æs incisis illustrata.* 2 vols. Londini (Sawbridge).

MARK, E. L., 1879–80. Bull. Mus. Comp. Zoöl. Harvard, **6**, 173.

MECKEL, J. F., 1815. *Handbuch der menschlichen Anatomie.* Erster Band. Halle and Berlin (Buchhandlungen des Hallischen Waisenhauses).

MIALL, L. C., 1911. *History of Biology.* London (Watts).

—— 1912. *The Early Naturalists: Their Lives and Work (1530–1789).* London (Macmillan).

MILNE EDWARDS, H., 1823. *Mémoire sur la structure élémentaire des principaux tissus organiques des animaux.* Paris (Migneret).

M'KENDRICK, J. G., 1888. Proc. Phil. Soc. Glasgow, **19**, 71.

MOLDENHAWER, J. J. P., 1812. *Beyträge zur Anatomie der Pflanzen.* Kiel (Wäser).

MONRO, A., 1726. *The Anatomy of the Humane Bones.* Edinburgh (Ruddiman).

MÜLLER, J., 1834. *Handbuch der Physiologie des Menschen für Vorlesungen.* Erster Band. Coblenz (Hölscher).

—— 1835. *Vergleichende Anatomie der Myxinoiden, der Cyclostomen mit durchbohrtem Gaumen.* Erster Theil. Berlin (Druckerei der Königlichen Akademie der Wissenschaften).

OKEN, (L.), 1805. *Die Zeugung.* Bamberg and Wirzburg (Goebhardt).

—— 1809. *Lehrbuch der Naturphilosophie.* Erster und zweiter Theil. Jena (Frommann).

—— 1843. Ibid. 3rd edit. Zürich (Schulthesz).

POLLENDER, A., 1868. *Wem gebührt die Priorität in der Anatomie der Pflanzen dem Grew oder dem Malpighi?* Bonn (Georgi).

PROCHASKA, G., 1779. *De structura nervorum. Tractatus anatomicus tabulis aeneis illustratus.* Vindobonae (Graeffer).

PURKINJE, —, 1838. Ber. Versamm. deut. Naturf. Aerzte, **15**, 174.

RÁDL, E., 1930. *The History of Biological Theories.* Oxford (University Press).

RASPAIL, F. V., 1833. *Nouveau système de chimie organique, fondé sur des méthodes nouvelles d'observation.* Paris (Baillière).

REMAK, R., 1855. *Untersuchungen über die Entwickelung der Wirbelthiere.* Berlin (Reimer).

RIES, E., 1943. Fort. Zool., **7**, 1.

SACHS, J. v., 1890. *History of Botany* (1530–1860). Transl. by H. E. F. Garnsey. Oxford (Clarendon Press).

SCHLEIDEN, J. M. [*sic*], 1849. *Principles of scientific botany: or, botany as an inductive science.* Transl. by E. Lankester. London (Longman, Brown, Green & Longmans).

—— M. J., 1838. Arch. Anat. Physiol. wiss. Med. (no vol. number), 137.

SCHWANN, T., 1838a. Neue Not. Geb. Nat. Heilk. (Froriep), **5**, col. 33.

—— 1838b. Ibid. 225.

—— 1839a. *Mikroskopische Untersuchungen über die Uebereinstimmung in der Struktur und dem Wachstum der Thiere und Pflanzen.* Berlin (Sander'schen Buchhandlung).

—— 1839b. (Contribution to Wagner, 1839.)

—— 1847. *Microscopical Researches into the Accordance in the Structure and Growth of Animals and Plants.* Transl. by H. Smith. London (Sydenham Society).

—— 1884. (Original words of Schwann, not known to be printed elsewhere, given by Frédéricq (1884).)

SEDGWICK, A., 1894. Quart. J. micr. Sci., **37**, 87.

—— 1895. Ibid., **38**, 331.

STIRLING, W., 1902. *Some Apostles of Physiology.* London (Waterlow).

STRASBURGER, E., 1880. *Zellbildung und Zelltheilung.* 3rd edit. Jena (Fischer).

SWAMMERDAM, J., 1737–8. *Biblia naturae; sive historia insectorum, in classes certas redacta.* With a preface by H. Boerhaave. Leydae (Severinum, Vander, Vander).

—— 1758. *The Book of Nature; or, the History of Insects: Reduced to Distinct Classes.* Transl. from the Dutch and Latin original edition by T. Flloyd. London (Seyffert).

TREVIRANUS, G. R. and L. C., 1816. *Vermischte Schriften: anatomischen und physiologischen Inhalts.* 1 Band. Göttingen (Röwer).

TURNER, W., 1890a. Nature, **43**, 10.

—— 1890b. Ibid., 31.

TURPIN, —, 1837. Ann. sci. nat., **7**, 207.

TYSON, J., 1870. *The Cell Doctrine: Its History and Present State.* Philadelphia (Lindsay & Blakiston).

—— 1878. Ibid. 2nd edit. Philadelphia (Lindsay & Blakiston).

VALENTIN, G., 1835. *Handbuch der Entwickelungsgeschichte des Menschen mit vergleichender Rücksicht der Entwickelung der Säugethiere und Vögel.* Berlin (Rücker).

VIRCHOW, R., 1859. *Die Cellularpathologie in ihrer Begründung auf physiologische und pathologische Gewebelehre.* Berlin (Hirschwald).

WAGNER, R., 1839. *Lehrbuch der Physiologie für akademische Vorlesungen und mit besonderer Rücksicht auf das Bedürfniss der Aerzte.* Leipsig (Voss).

WALDEYER, W., 1888. Arch. mikr. Anat., **32**, 1.

WEISS, P., 1940. Amer. Nat., **74**, 34.

WENZEL, J. and C., 1812. *De penitiori structura cerebri hominis et brutorum.* Tubingae (Cottam).

WHITMAN, C. O., 1893. J. Morph., **8**, 639.

WILSON, J. W., 1944. Isis, **35**, 168.

WOLFF, C. F., 1759. *Theoria generationis.* Halae ad Salam (Hendel).

—— 1774. Ibid. New edit. Halae ad Salam (Hendel).

WOODRUFF, L. L., 1939. Amer. Nat., **73**, 485.
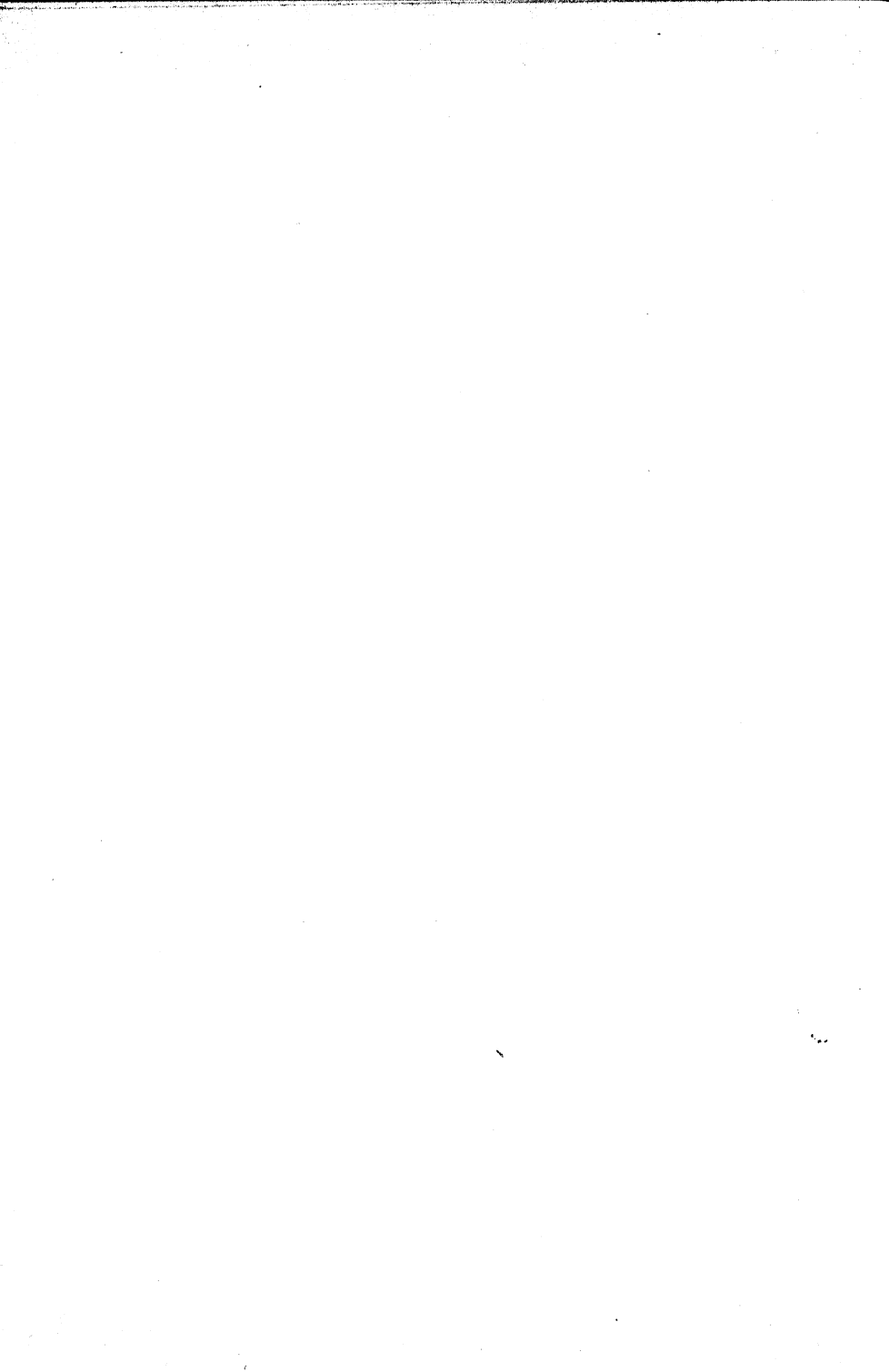
# The Pituitary in Normal and Parasitized Roach (*Leuciscus rutilus* Flem.)

BY

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**With one Plate**

### INTRODUCTION

THE occurrence of the plerocercoid stage of a tapeworm (*Ligula intestinalis*) in the body-cavity of the roach is accompanied by a marked regression of the gonads, and in view of the relationship of the gonads with the pituitary it seemed possible that this gland might also be involved. Histological changes in the pituitary have therefore been studied; in the first instance to determine whether there are any seasonal changes in the glands of normal roach, particularly in relation to the reproductive cycle, and then to ascertain whether the presence of the parasite induces further change.

The importance of the pituitary in relation to the gonads in teleosts is indicated by the stimulation of the gonads in *Pimelodus* by pituitary extracts of that species (Cardoso, 1934), and the degeneration following hypophysectomy observed by Vivien (1938, 1939) in *Gobius* and by Matthews (1939) in *Fundulus*. The problem could be advanced a further stage if some particular cell type can be associated with these gonad changes in the fish, since in higher vertebrates the acidophils of the distal (anterior) lobe appear to be especially associated with anabolism and growth and the basiphils with thyroid activity and gonad maturation.

### MATERIALS AND METHODS

Normal and parasitized roach were collected throughout the year from Thrybergh Reservoir, near Doncaster, where a high proportion of these fish are infected with *Ligula,* together with other normal fish from various sources in Yorkshire. The pituitary of the roach is attached to the brain by a delicate stalk and largely enclosed by bone and connective tissue, so that removal attached to the brain is impossible; some glands were dissected out separately therefore and their staining reactions compared with those of decalcified specimens still attached to the brain. The differences due to decalcification proved to be slight in this particular species, and to reduce the risk of damage the gland was left *in situ* and the lower half of the midbrain and the corresponding part of the roof of the mouth were removed together and treated as a unit. Various methods of fixation and staining were tried before the following routine was adopted: fixation in Bouin, decalcification in formol-nitric,

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double embedding by Peterfi's method after trimming the tissues, cutting serial longitudinal sections at 6 $\mu$ and staining by Mallory's (1936) modification of his own stain. The measurements of fish given are taken from the tip of the snout to the end of the solid part of the tail, not including the tail fin.

<div align="center">OBSERVATIONS</div>

### The Pituitary as a whole in Normal Adult Fish

The general structure of the pituitary can be seen in a median longitudinal section (Pl. I, fig. 1). The three divisions of the glandular (ectodermal) component are named from front to rear the anterior glandular region (anterior lobe of some authors), the middle glandular region (*Übergangsteil* or transitional lobe), and the posterior glandular region (intermediate lobe), for reasons given elsewhere (Kerr, 1942). As is usual in teleosts the lack of bounding connective tissue allows the cell types of each region to intermingle along the borders but the regions in themselves are histologically quite distinct. A brief description will first be given of these regions and of the cell types in which variation is irregular, followed in more detail by one of the single cell type recognized in this gland whose variations are sufficiently well marked and consistent to be associated with definite physiological states. The nervous lobe shows no changes that need be mentioned.

The anterior glandular region contains acidophils, basiphils, and chromophobes. The first are variable in size, often columnar, and rather solid cells whose closely packed granules hold both acid fuchsin and orange G to a moderate degree and soluble blue to a slight degree and hence stain a dark purplish-brown with Mallory; they have a moderate affinity also for azan and iron haematoxylin. The infrequent chromophobes look like degranulated cells of the same series. Neither show variations throughout the year nor any differences in parasitized fish. The basiphils are small rounded cells, few in numbers compared with the acidophils, usually light in colour but occurring in a complete range to very deep blue and showing considerable differences in numbers and granulation from one fish to another. It has not been possible to correlate these differences, however, with seasonal or other changes and the cells appear to be unaffected by parasitism. After Mallory these cells are similar in colour to the basiphils of the next region but after Anderson's (1929) modification of this stain have a dull amphiphil appearance, suggesting a separate type.

The middle glandular region again contains acidophils, basiphils, and chromophobes. These acidophils, however, are very small cells, with the granular cytoplasm frequently forming only a little cone on one side of the nucleus, and they have a strong affinity for acid fuchsin, azan, and iron haematoxylin and a very slight one for blue. They appear more brightly staining, therefore, and in that resemble the distal lobe acidophils of higher vertebrates. Their variations appear to be irregular, but their small size and large numbers make them an unsuitable type for study. The basiphils will be described

later. The chromophobes are not numerous and have very scanty and almost colourless cytoplasm. As Bock (1928) and others have pointed out there is nothing 'transitional', in Stendell's (1914) sense, about this region.

The posterior glandular region consists also of cells which from their reactions must similarly be called acidophils, basiphils, and chromophobes. The predominant type is a lavender-coloured basiphil, a cell, however, which has also a considerable affinity for orange G, azan, and iron haematoxylin. Neither they nor the occasional chromophobes show much variation. Scattered amongst them are acidophils with much the same staining reactions as those of the middle region but distinguishable by their larger size; these occur singly or in groups and vary from scarce to quite numerous. The variations appear to be haphazard and may be noticeable even in fish from a single catch. Again, the cells of this region do not appear to be affected by parasitism.

### The Basiphils of the Middle Glandular Region

These cells are scattered throughout the region with a general tendency to form irregular groups. They can be found in a complete series from cells with light-blue cytoplasm lacking discrete granules (about $5 \cdot 5 \mu$ in diameter) through cells of increasing bulk and with increasing numbers of rather small dense blue granules. This series is so complete that it does appear to represent a genuine sequence, in one or both directions; on the other hand, the linking of the degranulated basiphils to the smaller chromophobes (approximately $4 \mu$ in diameter) with their almost colourless cytoplasm may with less certainty be achieved through infrequent cells with reduced light-blue cytoplasm.

In describing the seasonal changes which can be seen in these basiphils a starting-point may be taken in April and early May, when the gonad is at its maximum and the shedding of eggs and sperm is about to begin. The basiphils are now also at their maximum. The light-blue cells are rare, almost all cells are deeply granulated, and the largest are oval or rounded and up to about $13 \mu$ in diameter (Pl. I, fig. 2). The high proportion of these very large cells is most characteristic of this time of year. The size and density of the basiphils give the impression in sections that their relative numbers, as compared with the acidophils, have greatly increased; actually this does not appear to be the case, but the small size and large numbers of the acidophils make accurate cell counts impossible. After breeding there is a regression of the basiphils and by late June or July they are at their least prominent. Then the proportion of degranulated and lightly granulated cells is much higher and the size of the largest granulated cells has fallen to approximately $8 \mu$ in diameter. After July there is a slow increase in granulation and cell size so that although there is some variation, the pituitaries of similarly sized fish in September and October are normally quite distinguishable from those of July, particularly in the size and number of the largest cells. There is a further increase during the winter, culminating in April and May.

## The Gonads of Normal Adult Fish

The corresponding changes in the gonads may be outlined briefly (see also Turner, 1919, for the perch, and Bullough, 1939, for the minnow). In the female the eggs are shed in May or June, by July the ovary is producing fresh oogonia, and many of these develop as primary oocytes during the summer. These oocytes in their primary growth phase, with characteristic basiphil cytoplasm, are common by September, and some occur in the secondary growth phase, with yolk droplets in a clearer cytoplasm. By late autumn both stages are abundant and many possess a vitelline membrane; little further change occurs during the winter and the final general maturation only becomes pronounced by spring. In the male in July the germ cells, characterized by their large lightly staining nuclei, are giving rise to numerous little groups of smaller denser spermatogonia; this continues into the autumn; in October primary spermatocytes appear and increase throughout the winter, but the final phases in the production of spermatozoa are again confined to the spring.

## The Pituitary and Gonads of Parasitized Fish

These fish have been caught from 7 cm. in length up to 22 cm., but the commonest size throughout two complete years has been 10–12 cm. with few fish ever exceeding 14 cm.

The effect of this parasitism on the pituitary is shown only by the basiphils of the middle glandular region, but there it is distinct and consistent. These cells differ from those of normal fish in the smaller maximum size that they normally attain (up to about $6 \cdot 5 \, \mu$ in diameter), in their much lower general level of granulation, and in the high proportion of small specimens with more or less clear blue cytoplasm. These differences are, of course, most marked in April and May (Pl. I, 3) when the normal basiphils are at their maximum, but they remain clear throughout the year. There is some variation from fish to fish even in the same catch, such as the appearance of an occasional more heavily granulated cell, but it is not sufficient to overlap the normal condition. Seasonal changes also are either absent or so reduced that they are obscured by this individual variation. The impression given by complete sections under lower power is that there has been a great reduction in the proportion of basiphils, but detailed observation under high power does not support this and it seems more probable that the proportion is not significantly altered.

The gonads of parasitized fish show a very uniform level of development at all times of the year, and this is true not only of the very small gonad which is typical of these fish but also of the occasional larger examples that do occur. The ovaries contain oogonia and primary oocytes which have developed up to about the end of their primary growth phase, the features of the secondary phase (loss of basiphility in the cytoplasm and the appearance in it of vacuoles and yolk droplets, and finally the vitelline membrane) have not been seen in the ovaries cut. In the testes the vast majority of the cells are germ cells with large clear nuclei and only a few small groups of the darker spermatogonia appear amongst them. The nuclei of the germ cells are more uniformly large

in size than in unparasitized fish, possibly owing to the lack of transformation stages towards spermatogonia, and the internal connective tissue framework gives only a very indefinite indication of a lobular organization. In both sexes, therefore, the gonads are comparable to those of spent fish in which all the later maturation stages are missing.

### Conditions in Immature Fish

The largest roach whose gonads are still immature in May are 7–8 cm. in length, and they show some features complementary to the foregoing. The ovaries with their oogonia and early primary oocytes resemble in sections those of parasitized or spent fish, although the gonad as a whole is very small; the testes, too, show a corresponding level of development except that the groups of spermatogonia are more numerous. The pituitary in both sexes is characterized by the small size of the basiphils of the middle region. These, with their reduced and lightly granular cytoplasm, closely recall the cells of parasitized fish; they occur in definite little clusters, foreshadowing the larger and less well-defined groupings seen in the adult.

### Thyroids of Adult Fish

The thyroids of normal and parasitized fish were cut in May, July, October, and January. The diffuse nature of the gland and the variations in different vesicles, even indeed in different parts of the wall of the same vesicle, make comparisons unusually difficult. Only very marked differences would show convincingly and such were not observed, but it must be emphasized that a considerable range of activity is possible without the manifestation of really distinct histological effects.

### Sticklebacks infected with Schistocephalus

The case of the stickleback (*Gasterosteus aculeatus* L.) infected by *Schisto-cephalus solidus* closely resembles that of the roach and *Ligula*—in the life-histories of the tapeworms concerned, in the occurrence of the plerocercoid in the body-cavity of the fish, and in the relatively enormous bulk of tapeworm to fish—and, since no information was elsewhere available, a brief examination has been made of such infected and normal fish. Bock (1928) did not find any typical middle-region basiphils in the normal pituitary at all; this may have been due to his employment of Susa since the use of this fixative, like decalcification, causes the basiphils to stain much like chromophobes. If the gland is fixed in Bouin, or better still corrosive formol, and dissected out with needles before sectioning, the basiphils can be seen as small, moderately staining, but not numerous cells, less well suited to the observation of changes than those of the roach, yet sufficiently clear to show any marked variations. No differences could be seen, however, between those of normal and parasitized fish. In the testes of infected fish cut in May there are appreciably more germ cells and spermatogonia still present than in normal fish, but there are at the same time large numbers of spermatozoa filling the lobules of the gland; full

sexual coloration also develops in parasitized fish and there seems little reason to doubt that they breed. In the ovaries the secondary growth phase of the oocyte, with yolk and vitelline membrane, certainly proceeds, but amongst the largest eggs there is a very marked degree of atresia (compare Vivien, 1939) and it is not possible to say whether eggs capable of fertilization are produced. However, it can be said that this is a case of much more balanced parasitism in which a pituitary of not noticeably altered appearance can carry the gonads towards the end of their maturation at least.

## DISCUSSION

The roach pituitary has its glandular component divided into the usual three regions found in teleosts—here called anterior, middle, and posterior glandular regions—and the division is based upon the cell types contained and not upon any precise connective tissue or other anatomical separation. Each region contains cell types which for descriptive purposes must be grouped under the three general headings of acidophils, basiphils, and chromophobes, but differences in size, shape, and staining reactions of the chromophils permit a clear differentiation. In the glands examined more or less distinct variations have been found amongst these chromophil cells from fish to fish and from season to season, but with the exception of those in one cell type they have been considered as too erratic or indefinite to sustain any conclusions.

The exceptional cell type is the basiphil of the middle glandular region (*Übergangsteil*, transitional lobe). When stained with Mallory these cells can at all times be found as a complete sequence from examples with non-granular pale-blue cytoplasm through stages with increasing amounts of small bright-blue granules up to large heavily loaded cells. Just before breeding in April and May the number of very large dense cells is at a maximum; after the breeding season the size and granulation decrease to a minimum in July with an increase in the proportion of degranulated cells; from then on there is a slow building-up until the pre-breeding condition is again attained. On this evidence an association between these basiphils and the maturation of the gonads can be suggested, and this suggestion is supported by the condition of the pituitary in fish parasitized by *Ligula*. Here the gonads are permanently reduced to about the level found in freshly spent fish—in the ovary oogonia and early primary oocytes, in the testis germ cells and some spermatogonia, with all the later maturation stages missing in each case—and the middle region basiphils in the pituitary are smaller and much less densely granular than those of normal fish at any time of year. Such variations as these cells show in parasitized fish appear to be individual and not related to seasonal changes, nor are they so extensive as to overlap those of normal fish. The remaining cell types in the gland show no changes as a result of the parasitism. The conditions found in the largest normal fish still immature in May are in good agreement; their gonads resemble those of parasitized and spent fish in the level of maturation attained, and their basiphils are of reduced size and granulation.

Comparisons with other fish cannot easily be made, since seasonal changes so far recorded for teleost pituitaries are few and difficult to relate to particular physiological states. The most complete is by Matthews (1936) for *Fundulus*; he divides the glandular component of the pituitary into two regions only, though Scruggs (1939) finds it composed of the usual three, and there is also some doubt as to which region Matthews's cell types actually belong. The cycles he finds, however, appear to be as follows, using the nomenclature for the regions adopted in the present paper: (*a*) a winter increase of middle region acidophils; these are large cells in *Fundulus* well adapted to show changes, whereas in the roach they are numerous and very small and regular changes were not detected; Matthews found middle region basiphils on the other hand rare and no changes were noted; (*b*) a more indefinite summer increase in the posterior region acidophils; these cells in the roach show considerable variations but of a kind too haphazard to be reliable; and (*c*) a summer and autumn increase in large basiphils which Matthews assigns to the posterior region but Scruggs, more plausibly, to the middle. Even so no parallel to the condition in the roach can be drawn. Comparison with Bock's (1928) results is even more difficult since in the middle region of the stickleback gland he found no typical basiphils at all and his seasonal changes are restricted to a spring increase in acidophil activity; finally, no comparison can be made with the results of Evans (1937). In the change from the yellow to the silver form of the eel, however, with the corresponding development of the gonads, Bernardi (1943) finds an increase in the middle region basiphils. In vertebrates higher than the fish there is some reason to associate the distal (anterior) lobe basiphils with the gonads. In seasonal changes (e.g. Hartmann, 1944, in the garter snake; Kayser, 1940, in the hedgehog) an increase in these cells coincides with gonad ripening; in development (e.g. Schooley and Riddle, 1938, in the pigeon) their final differentiation may await sexual maturity.

The removal of the pituitary in fish (Matthews, 1939, in *Fundulus*; Vivien, 1938, 1939, in *Gobius*) results after a time in an inability to form the later maturation stages of the reproductive cells or to maintain those already formed. Since this lack of later maturation stages characterizes the gonads in both sexes of these parasitized roach, it appears that here too the influence of the pituitary has been suppressed. Comparable results have been obtained from hypophysectomized mammals. In the male (e.g. Smith, 1930, in the rat) there is a clear similarity to the fish; in the female (e.g. Desaive, 1940, in the rabbit) stages in the ovary do not develop beyond that of the earliest liquid-producing follicles, a comparison with the fish is not here so simple, but both reproductive cells appear to be primary oocytes. The opposite experiment of removing the gonads to determine the effect upon the pituitary does not seem to have been performed on fish; in mammals, however (Ellison and Wolfe, 1934, 1935, in the rat), the result is an increase in the proportion of distal lobe basiphils, possibly followed by a characteristic degeneration. The reduced basiphils of the parasitized fish can hardly represent either of these

effects; in other words, the condition of the fish basiphils can hardly be itself a result of the regression of the gonads. In higher types also thyroid functioning appears to be largely under the same basiphil control, and the effects of thyroidectomy upon the pituitary resemble those of gonadectomy (Grobstein, 1938, in the newt; Brolin, 1946, in the rat), but it has not been possible to find definite parasitic effects on the roach thyroids.

The actual cause of the reduction of the basiphils in the roach is still undetermined, except that it is connected with the presence of the tapeworm or its waste products, but the effect is the virtual elimination of the gonad-stimulating hormone of the pituitary. From a brief investigation of the closely parallel case of parasitized sticklebacks it is clear that the influence of *Schistocephalus* on this fish is very much less severe, though there are indications in the ovaries of what may prove to be a slight effect of a similar nature. In the teleost, therefore, it is suggested that the middle region basiphils are particularly concerned in the maturation of the gonads—their influence becoming effective from the level of spermatogonia onwards in the male and of early primary oocytes in the female—and their maintenance at breeding-point, whether or not other factors determine the actual discharge of the gametes. These cells then would correspond physiologically to the basiphils of the distal lobe of higher types, a lobe to which the entire middle glandular region of the fish has a strong histological resemblance.

I am again in debt to Professor E. A. Spaul, D.Sc., for reading the manuscript and for his comments on the interpretations involved, and to Mr. G. Withem and Mr. W. H. Price of Doncaster and Mr. R. W. Ward of the Yorkshire Fishery Board for facilities and help in the collection of the fish.

## SUMMARY

1. A short description of the glandular component of the roach pituitary is given, from the point of view of the various cell types.

2. The seasonal variations are described in the basiphils of the middle glandular region (transitional lobe), the only cell type in this fish whose variations are sufficiently regular to be reliable, and a correspondence between these changes and the sex cycle is pointed out.

3. In roach parasitized by the plerocercoid of the tapeworm *Ligula* these basiphils are markedly reduced in size and granulation, whilst other cell types are not affected, and the gonads are also in a condition with all the later maturation stages missing.

4. The facts presented and a discussion of other work lead to the suggestions that it is these later stages of maturation which are under pituitary control in fish as in higher types, that the middle region basiphils are the principal cell type involved, and that these basiphils are comparable to the histologically similar basiphils of the distal lobe of later vertebrates.

## REFERENCES

ANDERSON, J., 1929. *How to Stain the Nervous System.* Edinburgh.
BERNARDI, C., 1943. Atti d. R. Instit. Veneto di Sci. Lett. ed. Art, **102,** 677.
BOCK, F., 1928. Z. wiss. Zool., **131,** 645.
BROLIN, S. E., 1946. Acta Anat., **2,** suppl. 3, 1.
BULLOUGH, W. S., 1939. Proc. Zool. Soc. A, **109,** 79.
CARDOSO, D. M., 1934. C. R. Soc. Biol., **115,** 1347.
DESAIVE, P., 1940. Arch. Biol., **51,** 587.
ELLISON, E. T., and WOLFE, J. M., 1934. Endocrin., **18,** 555.
——— ——— 1935. Ibid., **19,** 160.
EVANS, H. M., 1937. Proc. Zool. Soc. A, **107,** 483.
GROBSTEIN, C., 1938. Proc. Soc. Exper. Biol. and Med., **38,** 801.
HARTMANN, J. F., 1944. Amer. J. Anat., **75,** 121.
KAYSER, C., 1940. Ann. Physiol. **16,** 313.
KERR, T., 1942. Quart. J. micr. Sci., **83,** 299.
MALLORY, F. B., 1936. Stain Technol., **11,** 101.
MATTHEWS, S. A., 1936. Anat. Rec., **65,** 357.
——— 1939. Biol. Bull., **76,** 241.
SCHOOLEY, J. P., and RIDDLE, O., 1938. Amer. J. Anat., **62,** 313.
SCRUGGS, W. M., 1939. J. Morph., **65,** 187.
SMITH, P. E., 1930. Amer. J. Anat., **45,** 205.
STENDELL, W., 1914. 'Die Hypophysis Cerebri', in *Lehrb. d. vergl. mikr. Anat.* (Oppel), 8.
TURNER, C. L., 1919. J. Morph., **32,** 681.
VIVIEN, J.-H., 1938. C. R. Acad. Sci., **207,** 1452.
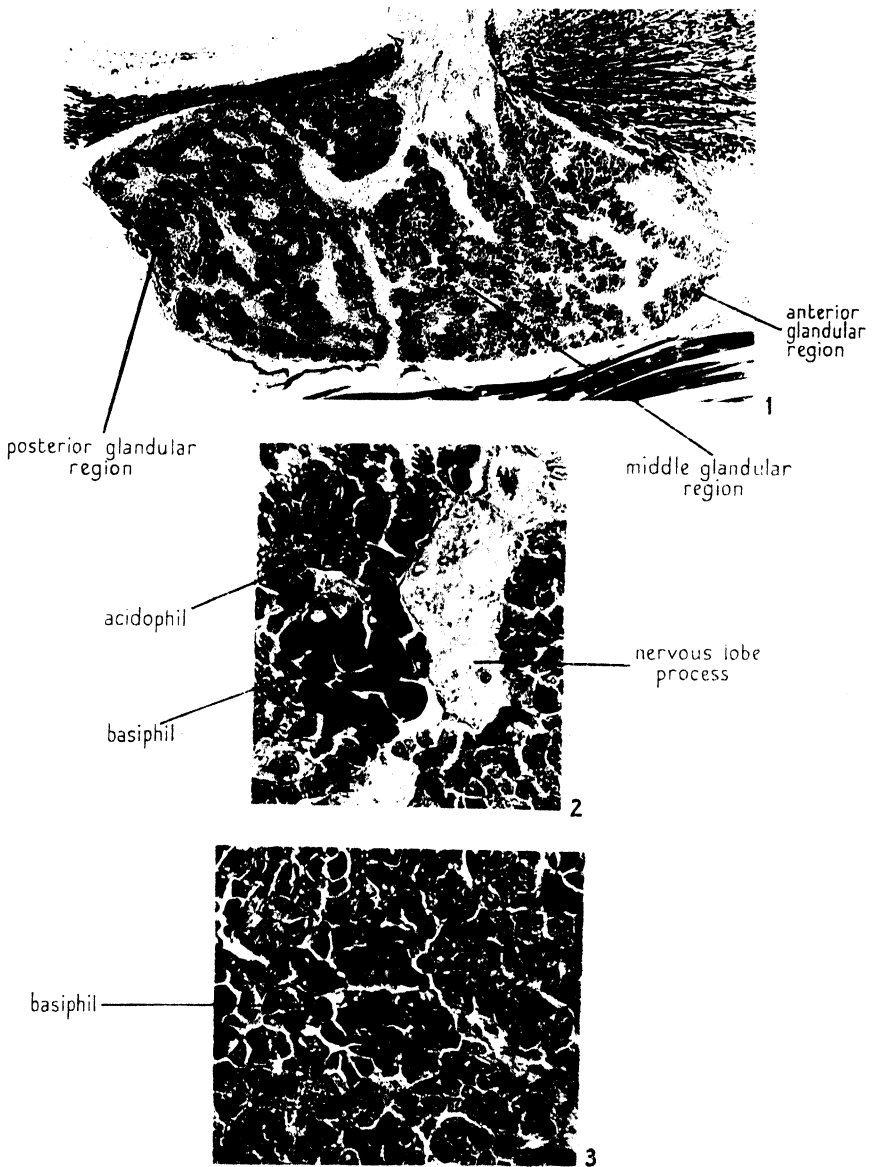——— 1939. Ibid., **208,** 948.

## EXPLANATION OF PLATE

### PLATE I

Fig. 1. Longitudinal section of pituitary of normal roach, 14 cm. in length. May. 6 $\mu$. Mallory. ×90.

A filter has been used to accentuate the groups of bright blue basiphils in the middle glandular region. In a corresponding photograph of the gland of a parasitized fish the reduced basiphils do not show up at all.

Fig. 2. Small group of full-sized basiphils in the middle glandular region of a normal fish, 13 cm. in length. May. 6 $\mu$. Mallory. ×550.

Fig. 3. Small group of reduced basiphils in the middle glandular region of a parasitized fish, 13 cm. in length. May. 6 $\mu$. Mallory. ×550.

anterior
glandular
region

1

posterior glandular
region

middle glandular
region

acidophil

nervous lobe
process

basiphil

2

basiphil

3

T. KERR.—PLATE I

# An Unusual Type of Muscle-fibre

BY

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**With two Text-figures**

I HAVE found an unusual type of muscle-fibre in the following species of the Serpulimorpha (Annelida, Polychaeta): *Serpula vermicularis* L., *Hydroides norvegica* (Gunnerus), *Vermiliopsis infundibulum* (Philippi), *Pomatoceros triqueter* L., *Protula intestinum* (Lamarck), *Spirorbis militaris* (Claparède), *Sabella spallanzanii* (Viviani), *Dasychone lucullana* (Delle Chiaje), and *Potamilla* sp. The parapodia of these annelids possess bundles of bristle-like chaetae and rows of hook-like chaetae, the bristles being notopodial in the thorax and neuropodial in the abdomen. The funnel-shaped protractor muscle of the bristle bundle in both thorax and abdomen is inserted round the base of the chaetal sac and has its origin on the body-wall round the opening of the sac. The muscle consists of a large number of discrete fibres extending all the way from origin to insertion; they are not held together by any connective tissue. These fibres were dissected out from *P. intestinum* and *S. spallanzanii* and examined alive, and were studied in sections of all the species listed above.

The central part of the fibre is composed of unstriped fibrils arranged in a hollow cylinder (Text-fig. 1*a*), surrounding an axial core of cytoplasm. The cylinder is covered by a sheath of cytoplasm which communicates with the axial cytoplasm by slits in the cylinder. The sheath of cytoplasm is expanded into two or more large frill-like membranes extending along the fibre (Text-figs. 1 *b* and *c*, 2). The folding of the membranes is presumably due to the contraction of the fibril cylinder. No more than one nucleus has been found in any of these long fibres. The nucleus is situated in a thickened region of one of the membranes (Text-fig. 1 *b, c, d*). The function of the membranes may perhaps be to present a large surface-area to the coelomic fluid. The protractor muscle is poorly supplied with blood-vessels and its fibres probably rely more on the coelomic fluid than on the blood as a source of oxygen, food, &c. Fox (1938) has pointed out that the body-wall musculature of sabellids has no special blood-supply and must similarly rely on the coelomic fluid.

Not only the fibril cylinder but also the frilled membranes of the living fibres are birefringent, and when they are highly magnified in polarized light the surfaces of the membranes show a fine pattern of delicate lines orientated in all directions and crossing over each other. In sections of these fibres stained by Heidenhain's 'Azan' method a thin blue sheath envelops the fibre

and covers the surfaces of its frilled membranes (Text-fig. 1*b*). These observations suggest that the muscle-fibre is covered by a sheath of connective tissue fibres comparable to the sarcolemma of vertebrate muscles.

Apart from the frilled membranes these muscle-fibres are variants of a type of fibre commonly found in annelids (Prenant, 1929) where the nuclei are often situated on the surface of the contractile part of the fibre, and where the
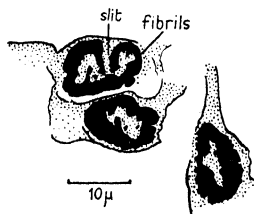


FIG. 1 *a*
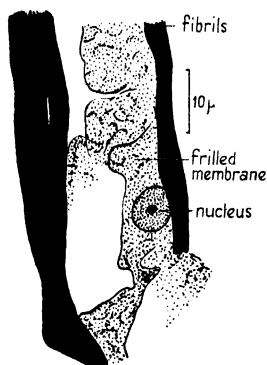
FIG. 1 *b*

FIG. 1 *c*
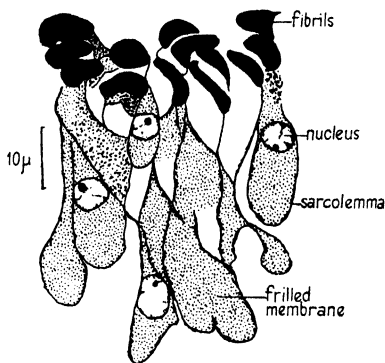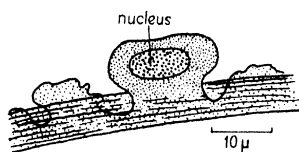
FIG. 1 *d*

TEXT-FIG. 1. Parapodial muscle-fibres. *a. Sabella spallanzanii.* T.S. three fibres. Iron haematoxylin. *b. Hydroides norvegica.* Oblique T.S. of a group of fibres. Azan. *c* and *d. S. spallanzanii. c.* L.S. part of two fibres. Iron haematoxylin. *d.* Free-hand drawing of part of fibre treated with methyl green-acetic.

fibrils are often arranged in cylinders with occasional openings putting the axial cytoplasm into communication with the peripheral cytoplasm. Goodrich (1942) isolated muscle-fibres from the 'hearts' of *Lumbricus herculeus* (= *Lumbricus terrestris* L.) and described 'processes' of the fibre which 'tend to form on either side a spreading extension with fringed edge and outstanding rounded lobes. These extensions seem to belong to a sheath of refringent material enclosing the true muscle-fibre, and it frequently shows fine closely set folds transverse to the main axis of the fibre, and giving it the deceptive appearance of a striated muscle.' I have confirmed this observation and found that the two 'extensions' are like the frilled membranes of the parapodial muscles of serpulids and sabellids.
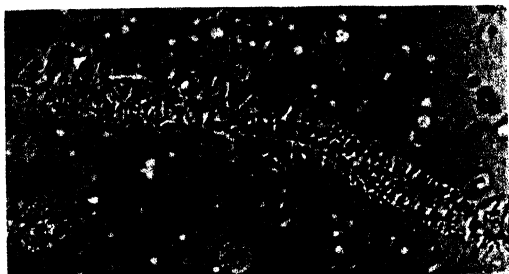
TEXT-FIG. 2. Photomicrograph of living parapodial muscle-fibre of *S. spallanzanii*. × 450.

## SUMMARY

The protractor muscles of the bristle-like chaetae of serpulids and sabellids consist of discrete fibres. Each fibre consists of a hollow cylinder of unstriped fibrils surrounded by a sheath of cytoplasm which is expanded into two or more frilled membranes extending along the fibre. The single nucleus lies in one of these membranes. The fibre is apparently ensheathed by a sarcolemma of fine connective tissue-fibres. Similar frilled membranes are present on the muscle-fibres in the walls of the 'hearts' of *Lumbricus terrestris*.

## REFERENCES

Fox, H. Munro, 1938. 'On the blood circulation and metabolism of sabellids.' Proc. Roy. Soc. B, **125,** 554.
Goodrich, E. S., 1942. 'A new method of dissociating cells.' Quart. J. micr. Sci., **83,** 245.
Prenant, A., 1929. 'Recherches sur la structure des muscles des annélides polychètes et sur leur sarcolyse.' Arch. Zool. exp. gén., **69,** 1.

# The Innervation of the Muscle-spindle

BY

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### With three Plates and thirteen Text-figures

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## Contents

## INTRODUCTION

WHEN Ruffini published his classical paper on the innervation of the muscle-spindle in 1898 it was widely maintained that the tendon-jerk was not reflex in nature but due to direct mechanical stimulation of the muscle. It has since been amply proved that the jerk is a reflex phenomenon, and it is commonly supposed (see, for example, Fulton, 1946) that the muscle-spindle is the receptor which excites the muscle contraction. According to Matthews (1933) the sensory fibres which terminate as Ruffini's 'flower-spray' endings in the spindles constitute the afferent side of the arc. However, though our knowledge of myotatic reflexes is now considerable it cannot be said that our information as to the precise form and disposition of nerve-endings in the muscle-spindle has made comparable progress. It is true that through the researches of Agduhr (1919, 1939) and Cuajunco (1932) we know that the innervation may be plurisegmental, and the development of

the innervation has been studied by Sutton (1915), Tello (1917, 1922), Cuajunco (1927, 1940), and Dickson (1940). Also the regeneration of spindle nerve-fibres has been followed by Huber (1900), Tello (1907), and Boeke (1916). But in the course of these investigations no fundamental analysis has been made of the complex innervation of the normal muscle-spindle.

The 'flower-spray' ending, to which such an important role has been assigned, has only been described in detail by Ruffini himself (1897, 1898). Though a few authorities have identified endings conforming with his description (Garven, 1925; Hinsey, 1927; Cuajunco, 1927, 1932; Hines and Tower, 1928; Denny-Brown, 1928a), their scrutiny has not been such as to provide us with additional information about the diameter of the fibre forming the ending or the exact relationship of the ending to the intrafusal muscle-fibres and the neighbouring 'annulo-spiral' nerve-ending. Such information is essential if any satisfactory correlation is to be made with the physiological data.

The experiments of Hinsey (1927), Boeke (1927), Hines and Tower (1928), and Cuajunco (1932) have convincingly demonstrated that the spindle receives a somatic motor innervation, but no complete analysis has been made of the number and distribution of the end-plates on the intrafusal muscle-fibres, or measurements made of the diameters of the nerve-fibres taking part in this innervation. In 1932 Tower was of the opinion that 'the rich confusion of nerve-fibres at the spindle poles' still awaited thorough analysis, and the recent electrophysiological study of Leksell (1945) on the 'gamma efferent' fibres emphasizes that this need still exists.

The present investigation of the innervation of the muscle-spindle was undertaken, firstly, as a necessary preliminary to a study of spindles reinnervated after nerve-injury and, secondly, in the hope that it might provide a solid basis for theories as to the mode of functioning of the end-organ.

## MATERIALS AND METHODS

The recovery of the rabbit's knee-jerk after crushing and after cutting and re-uniting the crural nerve has previously been followed (Barker and Young, 1947), and an attempt is being made to correlate the degree of recovery of the reflex with the extent of the reinnervation of the muscle-spindles. The normal innervation of the muscle-spindle has been studied chiefly in spindles from the rabbit's quadriceps so as to provide a control for the experimental investigation. Some preparations were also made of spindles from the rabbit's m. interossei, and the investigation was extended to spindles from the quadriceps of the cat.

Both silver and gold chloride techniques were used to impregnate the nerve-endings. Several silver methods were tried but that which gave the best results was de Castro's modification (1925) of one of the block impregnation methods of Cajal, the muscle being fixed in chloral hydrate, alcohol, and nitric acid. Various special modifications of the buffered silver nitrate method of Holmes (1943) were tried and proved useful for the demonstration of par-

ticular aspects of the innervation. The material was embedded in paraffin, and serial longitudinal sections cut at 20–25 μ.

Although preparations were made from most of the muscles belonging to the rabbit quadriceps, the muscle most often chosen was vastus intermedius I (Bensley, *Anatomy of the Rabbit*, 1938) because of its convenient size and shape and the fact that the direction of its fibres is such as to permit easy orientation for longitudinal sectioning.

The gold chloride technique used was that advocated by Gairns (1930) and within the limitations of the method it gave consistently good results. A few spindles from the rabbit's vastus intermedius were also teased out fresh in Ringer's fluid.

A spindle successfully impregnated by the gold chloride method gives an excellent overall picture of the innervation, and as compared with fresh specimens less shrinkage appears to have taken place than in material fixed for silver methods. However, the coloration is never uniform from one end of the spindle to the other, and it is impossible during teasing to leave the origin and insertion of the intrafusal muscle-fibres intact, or to be certain that all sources of nerve-fibres taking part in the innervation have been preserved. Moreover, for a detailed study of the relationship of the endings to the intrafusal fibres and their nuclei the method is inadequate. Preparations of spindles impregnated with silver do not suffer from these disadvantages. Whilst splitting up the end-organ into half a dozen or more 25 μ longitudinal sections makes it impossible to obtain a complete picture other than by reconstruction, it is only in this way that a thorough analysis of the innervation can be made. The majority of the spindles studied were therefore reconstructed from serial sections impregnated with silver, with gold chloride preparations providing a useful supplement for observing more general features.

Many of the ideas about the innervation were obtained from a single elaborate reconstruction of a spindle from m. vastus intermedius of the rabbit impregnated by de Castro's silver method. The spindle possessed two 'flower-spray' endings which lay one on each side of a central 'annulo-spiral' termination. It occupied nine 25 μ longitudinal sections, with the equatorial region occurring in five of these and portions of the small nerve-trunk innervating the spindle lying in a further seven sections. These sixteen sections were drawn at a magnification of 750 with the aid of a camera lucida using a $\frac{1}{12}$-in. objective. The nine sections containing the spindle were each covered on an average by about a dozen fields of view; a field of view once drawn was fitted together with the previous one and in this way a complete drawing made of the spindle as it occurred in each section. Altogether 130 fields of view were drawn and fitted together. Each region of the spindle was then reconstructed by collecting and fitting together all the relevant portions on to one major tracing, a process which necessitated a great deal of re-examination and re-drawing of certain portions of the spindle, notably the complex equatorial region. Each part of the reconstruction was re-checked when the final figure was drawn. From pole to pole the length of the spindle measured approximately

3·6 mm., so that at ×750 the complete reconstruction was over 8 ft. long. This has been reproduced in Plate I, reduced to a ninth of its original size.

This exhaustive study yielded much valuable information, but it was not considered necessary to make such elaborate reconstructions of other spindles. A considerable number of other spindles was examined and six spindles from the rabbit's quadriceps were reconstructed by the more rapid technique of drawing each section by eye and progressively building up a picture as the series was examined. As compared with the previous study these reconstructions were more in the nature of freehand sketches, but nevertheless they provided considerable additional information.
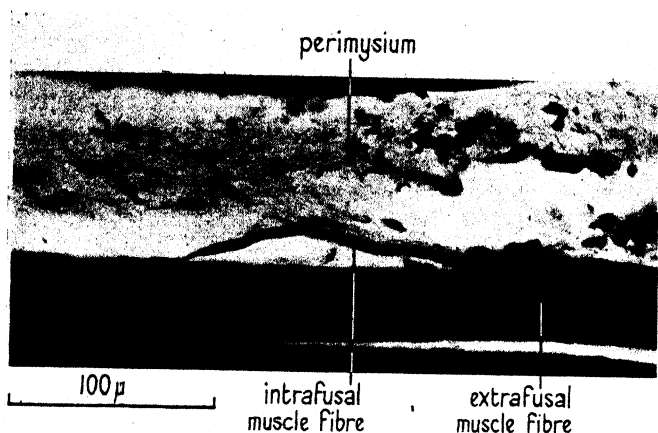
## THE INTRAFUSAL MUSCLE-FIBRE

It is necessary to consider the morphology of the muscle-fibres of the spindle before describing their innervation. In the classical descriptions (e.g. Sherrington, 1894; Ruffini, 1898) a distinction is made between the two poles of the spindle; there is said to be a proximal 'muscular' pole where the intrafusal muscle-bundle begins, and a distal 'tendinous' pole where the fibres taper off in long tendinous filaments. In between the two polar regions lies the equatorial region where the muscle-bundle courses through a fusiform space bounded by a thick lamellated capsule. According to Sherrington (1894) this space is lymphatic, for he succeeded in injecting it from the lymphatics of the leg. At the extreme proximal end of the spindle two or three muscle-fibres become invested by a common sheath of connective tissue. These are 'parent' intrafusal fibres, and as they approach the equatorial region each is said to split longitudinally into two or three 'daughter' fibres. Within the equatorial region the fibres become completely filled with centrally placed nuclei and form in most cases a distinct fusiform swelling. Whilst most of these features have been repeatedly described in the literature, it has never been convincingly demonstrated that the intrafusal fibres do undergo subdivision, and there is conflict of opinion as to whether or not the cross-striations are interrupted in that region of the fibre possessing the nuclear aggregation. A resolution of both these issues is essential to an understanding of the mechanism of the contraction of the intrafusal fibres and the effect this may have upon the sensory terminations.

The divided condition of the intrafusal muscle-fibres has been reported by the majority of observers from Kühne (1863) to Denny-Brown (1928a). Forster (1894) described how in the spindles of man the intrafusal fibres not only divided but became reunited again in such a way that their number in the equatorial region was from twice to four times that at the poles. Such division and re-fusion was also held to occur by Batten (1897). However, Kerschner long ago (1888) doubted whether subdivision of the intrafusal fibres occurred, and Baum (1900) failed to find any sign of it in a study of serial transverse sections of hedgehog spindles. Baum showed that the number of intrafusal fibres varied as a series was followed through from pole to pole, but found this to be due to the fibres being of different lengths, some

tapering off earlier, others later, in the polar regions. Cuajunco (1927), in his study of the development of the muscle-spindle of the pig, states that in all his preparations both of embryonic and adult material the intrafusal fibres preserved their individuality, the same number entering, passing through, and emerging from the capsule.
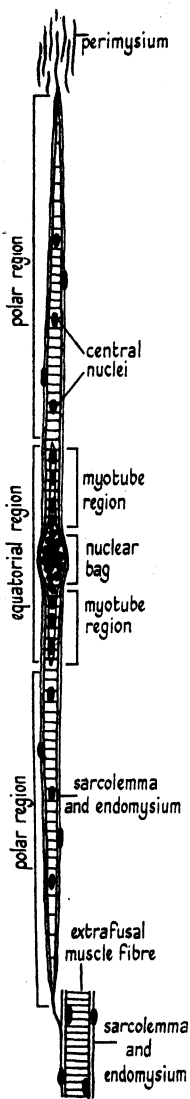
In my own preparations of rabbit muscle-spindles I have also found the intrafusal muscle-fibres undivided. The muscle-bundle is composed of 2–4 fibres and the same number can be counted throughout the greater part of the



TEXT-FIG. 1. Extreme end of an intrafusal muscle-fibre tapering off and becoming attached to endomysium of extrafusal muscle-fibre. Rabbit, m. vastus intermedius; L.S. 25 μ, de Castro silver method.

length of the spindle. It varies only towards the ends of the polar regions where the fibres, which vary in length, arise or insert at different levels. At these points they narrow down to mere filaments with wide spacing of their cross-striations, and ultimately they taper off into thin tendinous wisps. The wisps of the shortest fibres become merged with the connective tissue sheath enclosing the small muscle-bundle, and it is the places where this occurs that often present the deceptive appearance of one muscle-fibre subdividing into two. For example, in a polar region of one spindle a short intrafusal fibre tapered off in this way 0·7 mm. from the nearest end of the equatorial region, whilst two other longer members of the muscle-bundle tapered off 0·8 mm. and 1·1 mm. farther on, becoming merged with perimysium. The ultimate ends of the longer intrafusal fibres are either attached to tendon, perimysium, or the endomysial sheaths of various extrafusal muscle-fibres in the neighbourhood (Text-fig. 1).

There appears to be no constant feature which is characteristic of one pole and not the other, such as a particular mode of attachment, a considerable difference in length, or a difference in form of the muscle-fibres. Hence it is

perimysium

polar region

central nuclei

equatorial region

myotube region

nuclear bag

myotube region

polar region

sarcolemma and endomysium

extrafusal muscle fibre

sarcolemma and endomysium

TEXT-FIG. 2. Diagram of a single intrafusal muscle-fibre; each polar region has been shortened to about a third of its typical length.

impossible to distinguish between the 'proximal' and 'distal' poles of a spindle by mere inspection. These terms can only be used when it is possible to orientate the spindle according to the proximal and distal ends of the muscle. This can be done in sectioned material but rarely in gold chloride preparations since the orientation is usually lost during teasing. It is convenient to retain the terms for descriptive purposes and where they are used in this paper they merely indicate the orientation of the spindle within the muscle.

Ruffini (1898) maintained that the muscle-fibres at the extreme end of the proximal pole were 'always well apart from one another, and not closely bound together as in the rest of the spindle'. This is so when the fibres are attached to the endomysium of various extrafusal muscle-fibres, for not sharing a common origin they at first necessarily course apart from one another before running together as a bundle. In many spindles the proximal pole is attached in this way with the distal pole inserting on to tendon. But I have also observed spindles with both poles attached to extrafusal endomysium, and others where the distal pole has been attached in this way whilst the proximal pole has been attached to perimysial connective tissue.

In the polar regions elongated oval central nuclei occur at widely spaced intervals within the intrafusal muscle-fibres. In the equatorial region these nuclei become numerous and lie in a rod-like core of protoplasm so as to form a continuous chain. They then give way to a great mass of smaller spherical nuclei which at one point completely fills and often distends the body of the muscle-fibre (Text-fig. 2). It is proposed to call this aggregation of spherical nuclei the 'nuclear bag' (Bläschenspindel of Cilimbaris, 1910) and the portions on each side of it the 'myotube regions', for it is necessary to distinguish between these parts when describing the sensory innervation. As noted by Sherrington (1894) and Cuajunco (1927), in fixed preparations the diameter of an intrafusal fibre is less in its myotube regions than at the poles. Cuajunco suggests that this may be due to pressure exerted by the lymph in the equatorial region.

Each nuclear bag contains about forty or fifty spherical nuclei and is 100–150 $\mu$ long. The bags occur in a localized region of the equatorial area, usually towards one end, and do not all lie at the same level in the muscle-bundle but in such

a way that the proximal end of one such formation may begin opposite the distal end of another belonging to a neighbouring intrafusal fibre along-side. The number of nuclear bags in a spindle is always found to correspond with the number of intrafusal fibres present and provides a useful means of checking the number as observed in the polar regions. The only exception appears to be in rare forms of compound muscle-spindles where two or three equatorial regions occur in succession at widely spaced intervals along the same muscle-bundle. The region of the bag where the nuclei attain their maximum density appears to be completely devoid of cross-striations, the contractile substance having thinned away to leave the nuclear aggregation ensheathed only by the membrane of the muscle-cell ('sarcoplasmatic membrane' of Gutmann and Young, 1944) and its sarcolemmal covering. As Cuajunco (1927) states, 'If myofibrils are present at all in this segment they must be so few in number that the alternating dark and light bands are not shown.' It seems most probable that the intrafusal fibre is a double contractile unit, its contractile polar portions being separated by the non-contractile nuclear bag. The disposition of the motor innervation supports this view (see below).

## THE MOTOR INNERVATION

In every spindle examined the number of motor end-plates was approximately double that of the intrafusal fibres contained in the muscle-bundle, about half the number of end-plates being situated at each polar end. Variations in this arrangement occur when one polar half of an intrafusal fibre possesses more than one end-plate. I have never observed the condition figured by Denny-Brown (in Creed *et al.*, 1932, Appendix I) where motor end-plates are located at the proximal pole only, innervated by collaterals from neighbouring extrafusal motor fibres. It seems probable that in reptiles branches of ordinary motor fibres do contribute to the motor innervation of spindles (see Bremer, 1883; Perroncito, 1901; Boeke, 1927). If such a contribution is made in the rabbit the branching must occur at a considerable distance from the spindle for the nerve-fibres taking part in the motor innervation retain their individuality far back into the nerve-trunks which carry them.

The trunk carrying the majority of the fibres innervating the spindle usually approaches the equatorial region obliquely, coursing alongside one of the polar ends, most often the proximal. The motor fibres destined for this pole leave the trunk and come into relation with the muscle-fibres. The fibres innervating the other pole usually reach the capsule with the sensory fibres and run within the capsule walls, often with a 'flower-spray' afferent, before passing on to the polar region. The spindle nerve-trunk often contains a few extrafusal motor fibres which eventually leave to form end-plates on muscle-fibres near the end-organ.

The motor innervation of the intrafusal fibres distributed in this way may be augmented by a few additional fibres which leave a small trunk of extra-fusal motor fibres running near one of the poles of the spindle. In such cases

this trunk is always derived from the same mixed nerve-trunk which, lying in the neighbourhood of the spindle, supplies the rest of the spindle nerve-fibres and the surrounding extrafusal innervation.

In its general features the motor end-plate of a spindle is like an extrafusal motor end-plate (as described by Gutmann and Young, 1944), consisting of a hypolemmal termination ramifying upon a nucleated sole-plate. In silver preparations the ultimate twigs of the terminations end either as fine neurofibrillar brushes or as pointed tapers, but when treated by the gold chloride method the appearance is of blebs of various shapes for the most part connected with one another by fine filaments. A comparison between the form and disposition of the endings as seen in silver and gold chloride preparations leaves no doubt that the 'plate-endings' of Ruffini and the intrafusal motor end-plates are one and the same. The end-plates of the spindle are frequently large and often elongated so as to occupy a 50–70 $\mu$ stretch of muscle-fibre. The sole-plate may be confined to one part of the muscle-fibre or extend round the periphery to surround it almost completely. In some instances an ultraterminal twig from a large end-plate runs on for some distance to form a smaller plate on the same muscle-fibre. In such cases the endings may be regarded as two end-plates only in a limited morphological sense and are better regarded as one. On the other hand, when the smaller plate is located on another muscle-fibre it may be held to constitute a discrete termination. Nerve-fibres which enter the spindle and course without division to a single end-plate appear to be the rarest; typically the fibres divide so as to form several terminations. An end-plate frequently occurs which is innervated by branches of separate nerve-fibres or by two or three nerve-fibres which remain distinct from one another as far as they can be traced back from the spindle. It is possible that the fibres taking part in such multiple innervation are ultimately connected with a single nerve-fibre, though if this is so the branching must occur at a considerable distance from the spindle.

The motor innervation was worked out in full detail in the major reconstruction made of a spindle from m. vastus intermedius of the rabbit (see pp. 145, 146). Since examination of other spindles proved this to be a typical arrangement in most respects, it will be fully described. Text-fig. 3 shows the two poles of the spindle which possessed four muscle-fibres and altogether nine end-plates; five of these occurred on the proximal and four on the distal pole. The four intrafusal fibres could be traced through the equatorial region but in the polar regions it proved impossible to follow the course of each one with certainty. Their position in the muscle-bundle changed in such a way that although four muscle-fibres could always be detected in each section (except in the region of their origin and insertion) one was able to arrive at only an approximate idea of their complete course from one end of the spindle to the other. For this reason no strict statement can be made as to the number of end-plates possessed by any one of the muscle-fibres during its entire course, and in the figure the end-plates are not related to specific muscle-fibres. However, the
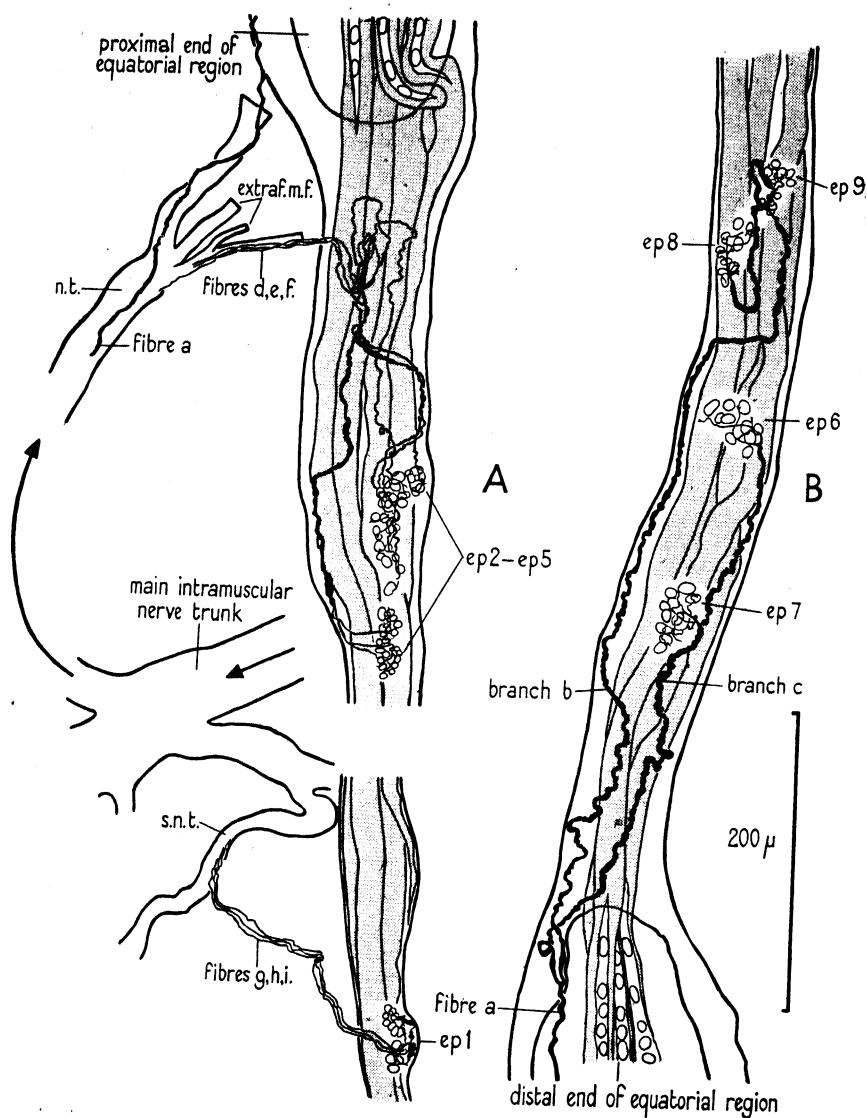
TEXT-FIG. 3. The two polar regions of the reconstructed rabbit's muscle-spindle showing the motor innervation (cf. Plate I). A, proximal pole; B, distal pole. Lettering described in text.

number and general disposition of the end-plates in this and other spindles studied strongly suggests that each polar half of an intrafusal muscle-fibre bears one, or sometimes more than one, motor end-plate, and since the two portions are separated by a non-contractile nuclear bag one would suppose that each functions as a contractile unit independent of the other.

The proximal pole (Text-fig. 3A) is innervated by six small myelinated fibres; three of these ($d, e, f$) are brought in by the nerve-trunk ($n.t$) which carries seven of the ten fibres innervating the spindle besides a number of extrafusal motor fibres (*extraf.m.f.*). The other three ($g, h, i$) leave a small nerve-trunk ($s.n.t$) of otherwise extrafusal motor fibres which is ultimately connected with the same main trunk from which the other spindle nerve-fibres were derived. The total diameter of each of these small fibres in this de Castro preparation was approximately 2 $\mu$ with an axon diameter of 1 $\mu$; measurements made of spindle nerve-fibres in fresh and gold chloride material suggest that they undergo a shrinkage of perhaps 50 per cent. in the fixed silver preparations. The three fibres $g, h, i$ enter the proximal pole about half-way along its length to form a single end-plate ($ep1$) whose sole wraps round the major part of the periphery of a muscle-fibre. These three nerve-fibres retained their individuality when traced back from the end-plate for a short distance into the nerve-trunk supplying them (a total distance of approximately 0·2 mm.); they could not be followed beyond this point and it is of course possible that they represent the branches of a single fibre which divides farther away from the spindle. The three fibres $d, e, f$ enter the pole near the proximal end of the equatorial region, having become segregated from the spindle nerve-trunk together with a sensory fibre. After a complex tangle over the surface of the intrafusal muscle-fibres the motor fibres course along the proximal pole to form four end-plates ($ep2$–$ep5$). The nature of these end-plates is shown in Text-fig. 4 where the nerve-fibres have been unravelled and the motor innervation of the two poles drawn out in schematic form. Fibre $d$ (Text-fig. 4A) is seen to run without division to form a simple spherical end-plate of medium size ($ep2$). Fibre $e$ gives rise to two twigs, one of which forms a small end-plate ($ep3$) close by the previous one and apparently on the same muscle-fibre, whilst the other contributes to a large elongated end-plate ($ep4$) on another muscle-fibre. Fibre $f$ makes an early division into two shortly after entering the spindle; one of these branches contributes to the elongated end-plate ($ep4$) whilst the other runs farther on to form an end-plate of its own ($ep5$). All these fibres increase considerably in diameter as they approach their end-plates, or immediately prior to breaking up into the final ramifications. A fibre possessing an average axon diameter of approximately 1 $\mu$ as it approaches the spindle may thus increase up to 3·4 $\mu$ shortly before terminating. A similar increase in diameter before terminating also appears to take place in extrafusal motor fibres.

The five end-plates on this proximal pole provide a typical example of the variety that is to be found in the motor terminations of muscle-spindles. The multiple innervation of intrafusal end-plates by apparently separate
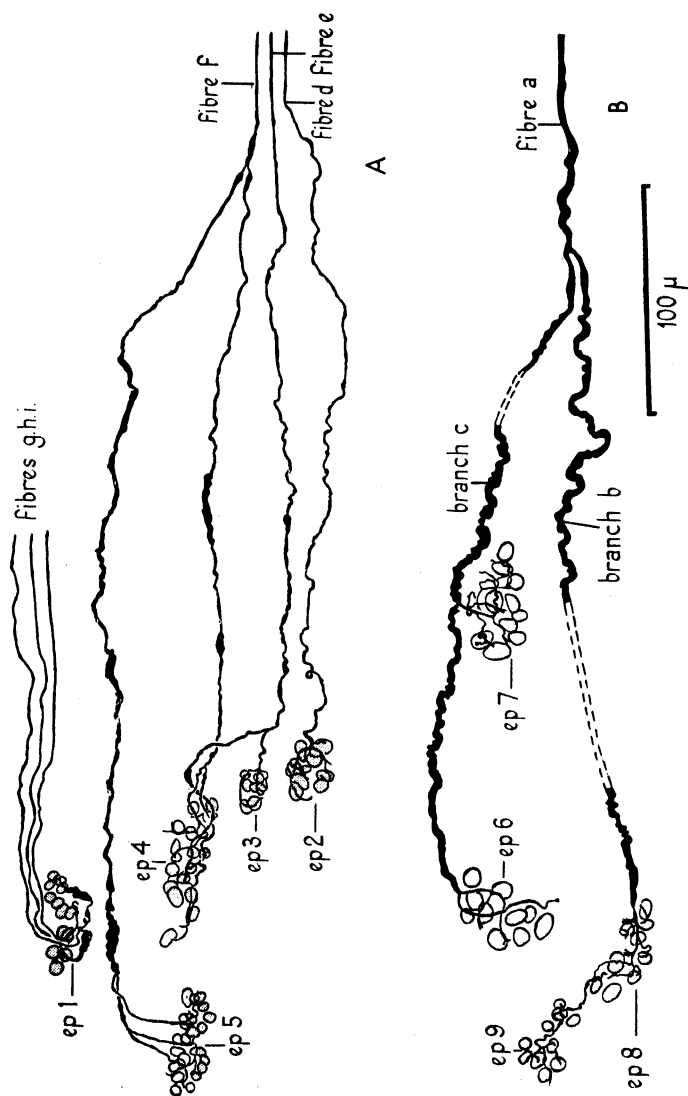
TEXT-FIG. 4. Schematic plan of the motor innervation of A, the proximal, and B, the distal pole of the reconstructed rabbit's muscle-spindle (cf. Text-fig. 3). Lettering described in text.

nerve-fibres and the occurrence of several distinct end-plates upon one polar half of a single intrafusal muscle-fibre are characteristic features, at any rate in the mammal. Cilimbaris (1910) found this to be the case in spindles from the extrinsic eye-muscles of sheep and various mammals, and both Dogiel (1902, mm. transversus and rectus abdominis of rabbit, monkey, and man) and Garven (1925, m. panniculus carnosus of hedgehog) observed several end-plates situated close together upon the same intrafusal muscle-fibre.
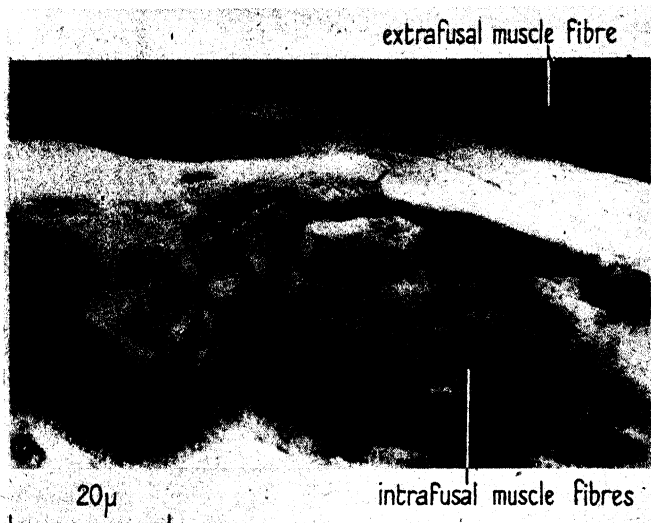


TEXT-FIG. 5. Intrafusal motor end-plate from distal pole of reconstructed rabbit's muscle-spindle (the same end-plate as *ep6* in Text-figs. 3 and 4). L.S. 25 μ, de Castro silver method.

The distal pole of this reconstructed spindle (Text-fig. 3B) was innervated by a single nerve-fibre (*a*) which was considerably larger than any of those innervating the proximal pole. Its average total diameter was 4·8 μ with an average axon diameter of 1·8 μ. It enters the spindle together with two sensory fibres at the proximal end of the capsule. Thence it runs partly within the capsule wall, partly in the lymphatic space, finally leaving the equatorial region at its distal end branching into two as it does so. The two branches (*b*, *c*) course down the distal pole, one of them (*b*) running for the most part in the connective tissue sheath surrounding the muscle-fibres; their diameters are on an average slightly greater than that of the parent fibre. Branch *c* courses for some distance before forming the large end-plate (*ep6*) photographed in Text-fig. 5. Shortly before this it gives off a small twig which immediately forms another large end-plate (*ep7*). Branch *b* runs beyond these two terminations farther down the distal pole to loop back on itself and then run on again to form a small end-plate (*ep8*) which sends off an ultraterminal twig to

another small end-plate (*ep9*) on a neighbouring muscle-fibre. The innervation of this pole is shown schematically in Text-fig. 4B.

It often happens that one pole of a spindle is innervated by one or at the most two relatively large motor fibres rather than by a number of smaller ones. Cilimbaris (1910) observed a large nerve-fibre supplying all eight end-plates of one pole, and Garven (1925) also noted the occurrence of large motor fibres innervating the spindle. In fixed silver preparations such fibres possess a total diameter of 4–5 $\mu$ whilst when treated by the gold chloride method they appear less shrunken and measure 6–7 $\mu$. The smaller intrafusal motor fibres are about half this size, being 2 $\mu$ or less in silver and 3–4 $\mu$ in gold chloride preparations. A motor fibre belonging to a spindle teased from fresh rabbit muscle was of these dimensions, having an average total diameter of 3·6 $\mu$. Owing to the usual increase in diameter of the nerve-fibres before terminating, measurements made during their course over the polar regions are apt to be misleading. A representative estimate of the average total diameter can only be obtained from measurements made at various points along the fibre as it is traced back from the end-organ. It is always difficult to follow their course for any considerable distance and only in favourable instances can one make a number of measurements. In his studies of the spindles of man and the dog, Batten (1897, 1898) failed to observe end-plates, but there is little doubt that the fibres he saw running to the polar regions were motor. His measurements varied from 2 to 6 $\mu$ in the two animals (Sihler preparations; chloral hydrate, acetic acid fixation) and are thus in agreement with the above figures. Hinsey (1934), reasoning from the data of Sherrington (1894) and Eccles and Sherrington (1930), supposed that muscle-spindles were innervated by motor fibres 7 $\mu$ or less in diameter (as seen in osmic preparations). Judging from my own measurements and those of Batten this does appear to be their order of size. Moreover, the fact that the intrafusal muscle-fibres were found to be innervated in many instances by both the smaller and the larger fibres within this range is in agreement with the work of Leksell (1945) on the group of small high threshold motor fibres designated by him 'gamma efferents'. He showed that there occurred a large increase in the afferent discharge from a muscle under some initial tension when these fibres were selectively stimulated, conduction in the larger 'alpha' fibres being blocked by pressure. However, an increase in the afferent discharge could also be produced when, without a pressure block, an efferent stimulus was applied which was sub-threshold for the 'gamma' fibres and activated members of the 'alpha' group only. From other experiments he concluded that in both cases this afferent activity arose from the muscle-spindles which thus appeared to be innervated by both the small 'gamma' efferent fibres and fibres larger than these belonging to the 'alpha' group.

An idealized conception of a muscle-spindle based upon the observations that have been made on the intrafusal muscle-fibres and their motor innervation is shown diagrammatically in Text-fig. 6. The spindle possesses three muscle-fibres which course from pole to pole without division. The extreme
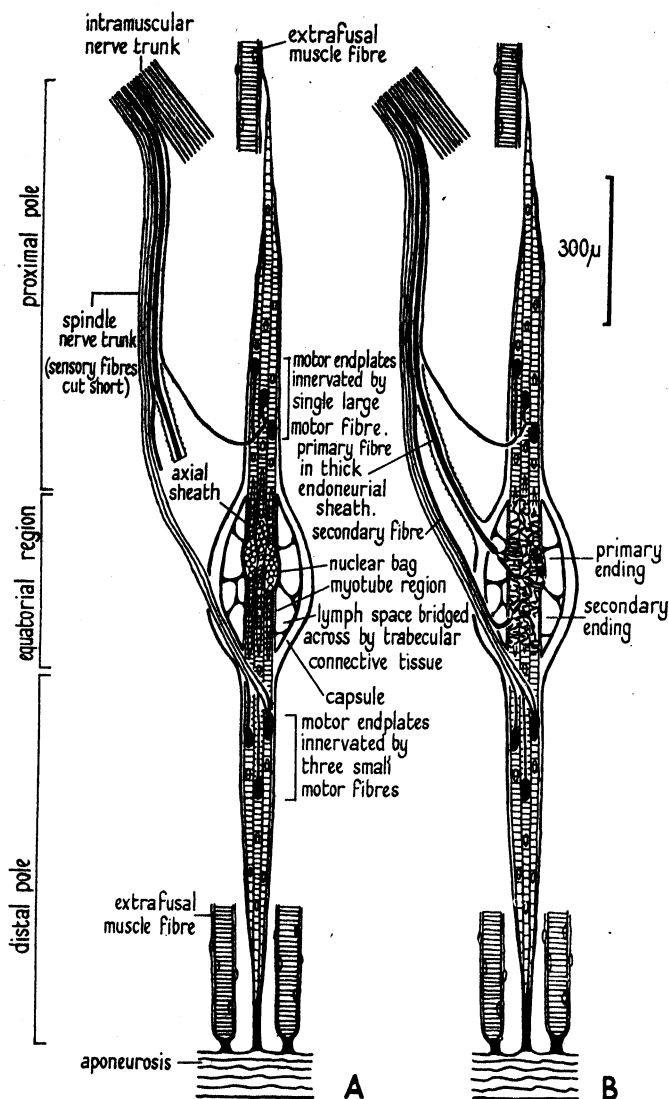
TEXT-FIG. 6. Diagrams of an idealized rabbit's muscle-spindle; polar regions shortened to about half their typical length. In A the motor innervation is shown, but the sensory innervation has been omitted to demonstrate the morphology of the equatorial region. Motor end-plates represented as black disks. B shows the same spindle with the addition of a sensory innervation comprising one primary and one secondary ending. Full description in text.

end of the proximal pole is attached to extrafusal endomysium, whilst at the distal pole the muscle-fibres insert on to an aponeurosis. In Text-fig. 6A the sensory innervation has been omitted in order to demonstrate the morphology of the equatorial region. A thin sheath of connective tissue ('axial sheath' of Sherrington, 1894) invests the bundle of muscle-fibres in this region and is united to the inner wall of the capsule by delicate septa which bridge across the lymph space. Each muscle-fibre possesses a non-striated nuclear bag, and to either side of the bag has the appearance of a myotube. In the two polar regions the muscle-fibres each bear one end-plate so that the spindle has a total of six end-plates with three at each pole. An exact 3 : 3 ratio is shown, for it serves to emphasize the fact that in all probability the two polar halves of an intrafusal muscle-fibre function as independent contractile units. The end-plates at the distal pole are innervated by three small motor fibres, and those at the proximal pole are supplied by a single rather larger fibre. Spindles with both poles innervated by large motor fibres have not been observed; in many spindles both poles are innervated by small fibres. Leksell's experiments suggest that the large fibres belong to the 'alpha' group, and that the smaller ones are 'gamma' efferent fibres. In the figure four motor fibres are shown innervating a spindle possessing three muscle-fibres. The number of motor fibres innervating a spindle varies considerably; it always exceeds the number of intrafusal muscle-fibres and is often approximately double this number. Branching of intrafusal motor nerve-fibres has only been observed after the fibres have entered the spindle; they have always remained unbranched in the spindle nerve-trunk when this has been traced back to its derivation from a main intramuscular nerve-trunk.

In Text-fig. 6B the same spindle is shown with the addition of an equatorial innervation comprising two sensory terminations. The nature of the sensory innervation is described below.

## THE SENSORY INNERVATION

### *Introduction*

The sensory endings of the muscle-spindle are amongst the most complicated of all peripheral terminations; Sihler (1895) has described them as *ein hoffnungsvernichtender Anblick*. The detailed investigations of Ruffini (1893–8) provide the most complete analysis that has yet been made. Huber and de Witt (1898), Dogiel (1902), Cilimbaris (1910), and Tello (1922), amongst others, have supplied information about other mammals and vertebrates, but it has yet to be correlated with Ruffini's findings. Before making such a correlation and presenting the results of the present investigation it is necessary to consider Ruffini's work in some detail.

He used the gold chloride method and made the majority of his observations on muscle-spindles of the cat. He distinguished (1897, 1898) between two types of equatorial sensory termination, a primary or 'annulo-spiral' ending, and a secondary or 'flower-spray' ending. The primary ending is supplied by a large nerve-fibre which subdivides just before penetrating the capsule. After further subdivision

the ultimate branches come into contact with the intrafusal muscle-fibres and ramify so as to encircle them with a series of rings and spirals. The endings are composed of wide flat ribbons and their turns around the muscle-fibres become more widely spaced apart towards either end of the central area of termination. At these extremities the rings and spirals give way to more irregular 'rounded clavate or leaf-like figures'. Ruffini noted that some primary endings were altogether less regular in form with only a few rings and spirals, 'the greater part showing S or C forms intercalated among forked, hooked, or comma shapes'.

The secondary ending is described as being formed by a medium-sized fibre which always enters the capsule at some distance from the entrance of the primary fibre and is usually carried in a separate nerve-trunk. Subdivision occurs only after the capsule has been penetrated, and the branches formed are never numerous. The endings always lie close to the primary ending within the equatorial region but are more dispersed. They are applied 'both around and upon' the muscle-fibres and usually consist of a great number of varicosities of various shapes joined together by fine filaments, the general arrangement often resembling a spray of flowers. Ruffini observed that some of the endings possessed 'a typical feature recalling the less common S and C claspers of primary endings'.

On the basis of the presence or absence of the secondary endings Ruffini classified the muscle-spindles of the cat into three types:

1. *Muscle-spindles with complex nerve-ending.* These possessed two secondary endings in addition to a single primary ending. The secondary endings might both be located on one side of the primary or lie on each side of it. Ruffini found spindles of this type to be the most common.

2. *Muscle-spindles with intermediate nerve-ending.* These possessed one secondary and one primary ending. The secondary ending was located on either the proximal or distal side of the primary. Ruffini found this type to be the least numerous.

3. *Muscle-spindles with simple nerve-ending.* These possessed a primary ending only, usually of the more irregular type. The muscle-fibres of these spindles were short and few in number.

A comparable study of the afferent innervation as it occurred in the rabbit has confirmed many of Ruffini's observations. Particular attention was paid to the secondary fibres and their terminations which are supposed to constitute the afferent side of the stretch reflex arc. Their distribution was found to be approximately as Ruffini maintained, but their form was not 'flower-spray' in the rabbit but 'annulo-spiral', differing only in minor details from that of the primary endings.

### The Sensory Innervation of the Rabbit

*Types of Equatorial Innervation*

The sensory endings are confined to the nuclear bags and myotube regions of the intrafusal muscle-fibres in the equatorial region. The primary ending is always associated with the nuclear bags, and its ramifications usually entwine only small portions of the myotube regions. In some spindles, however, particularly those in which it is the only sensory ending present, it may be

more extensive and occupy large areas of the myotube regions as well as the nuclear bags. The secondary endings are confined to the myotube regions; some of their ramifications may encroach upon the ends of the nuclear bags but never to any great extent. The two types of termination usually lie close together but never become continuous. Moreover, the fibres supplying the endings remain separate when traced back into the main nerve-trunk from which they were derived. Their course has been followed from nerve-trunk to equatorial region in a number of spindles, in one case for a distance of 3 mm., in another for 5 mm. Throughout their approach they remain as individual fibres, the primary large ($8$–$12$ $\mu$), the secondary of medium size ($6$–$9$ $\mu$), and there can be little doubt that this independence persists in the muscular nerve-trunks in view of Matthews's (1933) recordings from single-fibre preparations demonstrating two types of spindle afferents, and Tello's observation (1922) that the secondary fibres reach the spindle considerably later during development than the primary ones.

The disposition of the sensory endings in the spindles examined conformed to Ruffini's classification. The primary ending may be the only sensory termination present (simple type of spindle), or it may be accompanied by one secondary ending (intermediate type of spindle), or by two (complex spindle). The different types may occur within the same muscle; thus in six spindles from the vastus intermedius of a rabbit the primary ending was accompanied by one secondary ending in three spindles (intermediate type), by two secondary endings in two spindles (complex type), and in one it was the only sensory ending present (simple type). Spindles with one primary and one secondary ending occurred most frequently in the samples taken. Thus of sixteen spindles from mm. vastus intermedius and vastus lateralis of six different rabbits, ten were of this type; four others were complex with two secondary endings in addition to the primary, and the remaining two were simple with a primary ending only. A further six spindles taken from the mm. interossei of one rabbit were composed of three of the intermediate type, one of the complex, and two of the simple. In that the choice of the spindles providing these data was governed entirely by the hazards of the histological techniques employed, it can be said to be random. Providing that the impregnation was sufficiently successful to permit an analysis of the sensory innervation, the spindles were selected as serial sectioning revealed them, or as they were found by teasing.

Various types of equatorial innervation are diagrammatically shown in Text-fig. 7. Three varieties of simple spindle, where only the primary ending is present, are shown at A, B, and C; the main difference between them is the extent of the area occupied by the primary ramifications. In all types of spindle this appears to vary according to the distance between the nuclear bag region of the intrafusal muscle-bundle and the first subdivision of the primary fibre. The most usual condition is for this distance to be short, the fibre penetrating the capsule, subdividing within the lymphatic space, and almost immediately making contact with the nuclear bags. The major part of
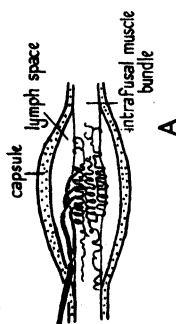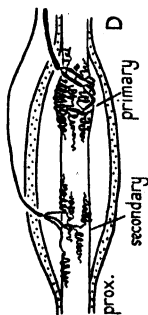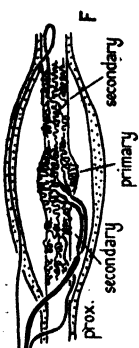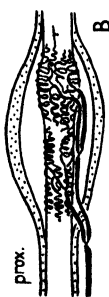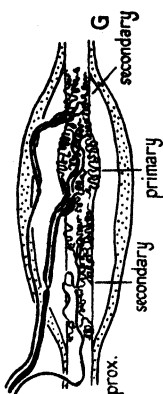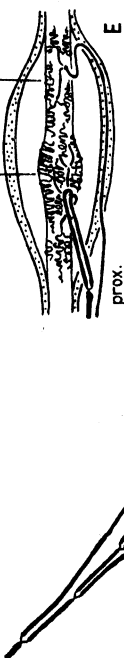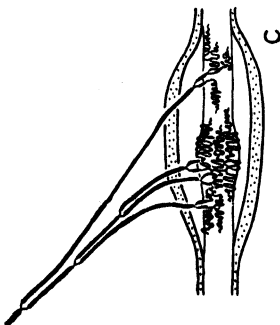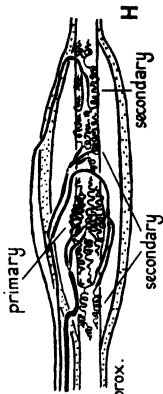
TEXT-FIG. 7. Diagrams of the equatorial regions of eight rabbit muscle-spindles illustrating various types of sensory innervation. Intrafusal muscle-bundle shown in outline only and area of nuclear bags indicated by exaggerated swelling. Spindle c from m. interosseus, the rest from m. vastus intermedius of quadriceps. B, D–H diagrammatized from reconstructions made from silver preparations; the proximal end (*prox.*) lies to the left in each case. A and c diagrammatized from gold chloride preparations; identification of proximal and distal ends not possible. See text.

the ending is then situated around the bags, with a few ramifications spreading out around the myotube region on either side. The primary ending of the simple spindle in Text-figs. 7A and 9 is of this type, and this is its typical relation to the muscle-bundle when accompanied by secondary endings. In the other types of simple spindle shown (Text-fig. 7B and c) the primary fibre undergoes its first subdivision some distance away from the nuclear bags and the area of termination is greater. The point of division occurs just before the capsule is penetrated in the spindle illustrated in B. The two branches enter the proximal end of the equatorial region; the nuclear bags, however, lie at the distal end, and between them and where the branches enter there is an extensive myotube region. One of the branches forms a group of ramifications around this region, whilst the other runs on to form another group around the nuclear bags. In the third type of simple spindle shown (7c) the primary fibre branches exceptionally early, 320 $\mu$ before penetrating the capsule. Moreover, before the capsule wall is reached one of the branches undergoes further subdivision so that altogether four separate branches enter the equatorial region. They form an extensive ending around the nuclear bag area and its myotube regions on either side. In these simple types of spindle the equatorial region is always smaller than when additional secondary endings are present, being 250–350 $\mu$ long as compared with a length of 500–600 $\mu$ in complex spindles.

Text-fig. 7D and E shows two varieties of the intermediate type of spindle where there is one secondary ending in addition to the primary; the idealized muscle-spindle shown diagrammatically in Text-fig. 6B is also of this type. The distribution of the two types of ending varies according to where the nuclear bags of the muscle-fibres are located in their course through the equatorial region. They usually occur towards one end so that the myotube regions are short on one side of them and long on the other. The secondary ending occupies the longer of the two myotube regions and may lie either proximally (as in 7D) or distally (as in 7E) to the nuclear bags and primary ending. In the spindle shown in 7E there is less difference in length between the two myotube regions for the area of nuclear bags is located more towards the middle of the equatorial region. The slightly shorter proximal myotube region receives several ramifications from the primary ending which is extensive, early subdivision of its nerve-fibre having occurred.

The secondary ending may intercalate with the primary (as in Pl. II, fig. 5) or lie well apart from it (as in Text-fig. 7D). In two instances I have observed a portion of the secondary ending extending for a short distance on to one pole, but usually the terminations are confined within the equatorial region. The primary and secondary fibres are always carried in the same nerve-trunk but course apart from each other when this breaks up at a varying distance from the equatorial region. The primary fibre usually penetrates the capsule at one end and the secondary fibre at the other or more towards the middle. The secondary fibre often courses for a considerable distance within the walls of the capsule before finally entering the lymphatic space.

Three varieties of the complex type of spindle are shown in Text-fig. 7 F, G, and H. Here two secondary endings accompany the primary one; the most frequent arrangement is for the secondary endings to lie one on each side of the primary, which entwines the nuclear bags in the middle of the equatorial region (7F). When one myotube region is short and the other long, one of the secondary endings is accordingly less extensive than the other (7G, see also Pl. I). Alternatively, the secondary endings may lie in series with one another on the same side of the primary ending. A variation of this arrangement is shown in 7H where one of the secondary fibres divides so as to terminate not only on the distal side of the primary ending, where the other secondary ending is located, but also on the proximal side.

In the major reconstruction made of the complex spindle shown in Plate I it was possible to map out the areas covered by the three endings. Each of the four members of the intrafusal muscle-bundle could be followed from one end of the equatorial region to the other, a distance of approximately $560\mu$. Their equatorial portions have been accurately drawn in Text-fig. 8, where the four muscle-fibres ($a$, $b$, $c$, $d$) have, as it were, been teased apart from one another and arranged side by side. In representing the muscle-bundle in this manner the original alignment of each fibre relative to the others has been retained. The fibres vary in diameter as Cuajunco (1927) has described in the pig; the nuclear bag is greatest in the fibres of largest diameter (cf. fibres $a$ and $b$). The areas occupied by the terminal ramifications of the three endings are shown by differential shading. Of the two secondary endings which lie on either side of the primary, the proximal one is the more extensive owing to the location of the nuclear bags within the distal half of the equatorial region. The primary ramifications are seen to be confined chiefly to the nuclear bags though they also extend over the distal myotube portions of the muscle fibres $a$ and $b$. At four places they overlap with secondary terminations but not to any great extent. The two secondary endings for the most part occupy their respective regions on either side of the primary ending, being distributed mainly over the myotube portions but with encroachments here and there on to the end of a nuclear bag. A ramification of the distal secondary ending, however, extends over to the proximal myotube portion of one of the muscle-fibres ($d$). Here it overlaps with part of the primary ending and also with a part of the proximal secondary ending.

This map of the distribution of the three endings shows that though the areas they occupy are closely intercalated, the extent to which they actually overlap is small. The muscle-fibres are shown with marginal indentations occurring from place to place; these are constrictions caused by the nerve-endings whose form will now be described.

### The primary ending

The primary ending is always derived from one nerve-fibre; I have never observed two independent fibres contributing to the termination as Ruffini (1898) found in some spindles of the cat. As noted above (p. 159), the
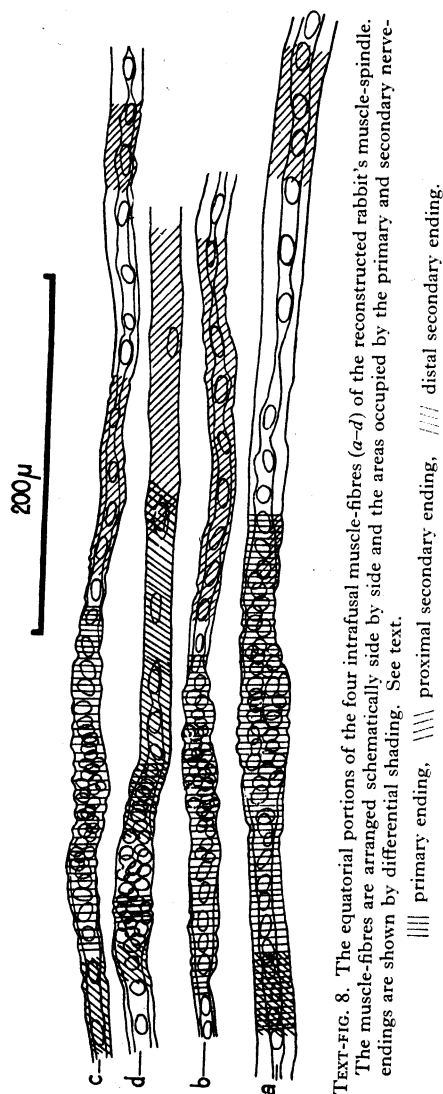
TEXT-FIG. 8. The equatorial portions of the four intrafusal muscle-fibres (*a–d*) of the reconstructed rabbit's muscle-spindle. The muscle-fibres are arranged schematically side by side and the areas occupied by the primary and secondary nerve-endings are shown by differential shading. See text.

||||| primary ending,  \\\\ proximal secondary ending,  ///// distal secondary ending.

area covered by the ending varies according to whether the first subdivision of the primary fibre occurs before or after it penetrates the capsule. When the division occurs after, the ramifications are restricted chiefly to the nuclear bags of the muscle-fibres, with only a few extensions around the myotube regions (Text-fig. 9B). This type of distribution will be considered first since it is that which most frequently occurs.
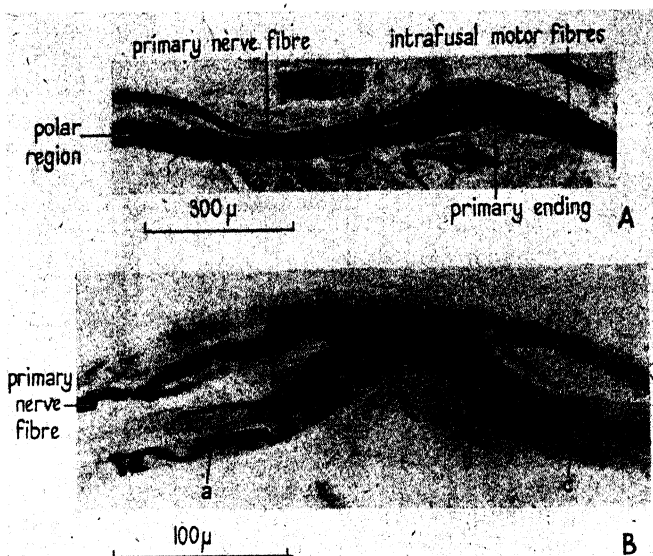


TEXT-FIG. 9A. Simple spindle with sensory innervation composed of one primary ending. B. Primary ending of same spindle. The ending is confined chiefly to the nuclear bags of the intrafusal muscle-fibres with a few ramifications (*a, b, c*) extending around the myotube regions on each side. Rabbit, m. vastus intermedius; Gairns gold chloride method.

The first division of the primary fibre is usually dichotomous, though sometimes three branches are formed. Further subdivision rapidly ensues to give rise to branches of the third and fourth order which form the terminal ramifications. These branches remain myelinated until the 'preterminal node' of Ruffini (1898) is reached and the terminations are formed. Each branch usually gives rise to one system of ramifications, though occasionally it may form two. In some instances two branches will contribute to form one system. The endings consist of a series of half-rings and spirals; complete rings are rare. They are closely set together around the middle portions of most of the nuclear bags, but to either side they are more widely spaced apart. Thus when entwining the most densely nucleated region the turns of a spiral will be vertical and tight together, but when coiling around one side of a bag and extending into the myotube region the turns are loose and run diagonally (Pl. III, fig. 10). The few terminations which extend for any distance around

the myotube regions are usually of an irregular or clasping form (Text-fig. 9B, *a*, *b*, and *c*).

A ramification system may be confined to one member of the muscle-bundle, or entwined chiefly around one but with a minor part around another, or more or less equally spread over and interlocked around two neighbouring intrafusal muscle-fibres. Each member of the muscle-bundle is entwined to a greater or lesser extent. The axoplasm wraps around the muscle-fibres in the form of a wide flat ribbon as Ruffini described. In silver preparations the ribbons have a coarse neurofibrillar appearance and the ultimate end of any termination is by means of a brush-like neurofibrillar expansion. The endings lie underneath the sarcolemmal and axial sheaths and are closely applied to the 'sarcoplasmatic membrane' of the muscle-fibres. In fixed silver preparations this relationship is particularly well shown, for where the endings curl round the muscle-fibres they constrict them so as to form marginal indentations, leaving the connective tissue running above (Pl. III, fig. 6). It seems probable that such constrictions are due to a difference in the degree of shrinkage during fixation undergone by the axoplasm of the nerve-endings and the sarcoplasm of the muscle-fibres. The muscle-fibres are not constricted in this way in fresh preparations, where in favourable instances it is possible to see spiral terminations, and in gold chloride preparations the constrictions are also absent.

In the major reconstruction made of the complex spindle (Pl. I) the primary ending was found to be composed of nine separate ramification systems. These have been schematically arranged in Text-fig. 10 so as to demonstrate the morphology of the ending to the best advantage. The figure is schematic in that the muscle-fibres and nerve-branches have been broken from place to place in order to reveal all parts of the ending. The portions thus disintegrated have been accurately drawn, and in arranging them in the form of a plan their original relationships with each other have been preserved as far as possible. The majority of the ramifications are seen to be entwined around the largest muscle-fibre present in the bundle (*a*), and this is a typical condition. The muscle-fibre *d*, on the other hand, receives only a few half-rings and the greater part of its nuclear bag is not entwined. At *e* and *f* the interlocking type of ramification system binds two members of the muscle-bundle together by means of a number of half-rings and spirals. At *g* and *h* the ramifications are distributed from a central rib somewhat as Ruffini figures (1898, fig. 5). The terminations *j* and *k* are of the irregular clasping type which is characteristic of the extremities of the ending; *j* contains a small ring lying on the surface of the muscle-fibre. At *l* three half-rings are closely set together around the middle of a nuclear bag, whilst at *m*, which lies adjacent, the terminations are at first set closely together and then form a series of loose spiral turns extending away from the nuclear bag. Spiral systems never exceed four or five turns, and when they attain this number the coils are always widely spaced and found to one side of a nuclear bag, as figured here and shown in Pl. III, fig. 10. Altogether the primary ending entwined the four muscle-
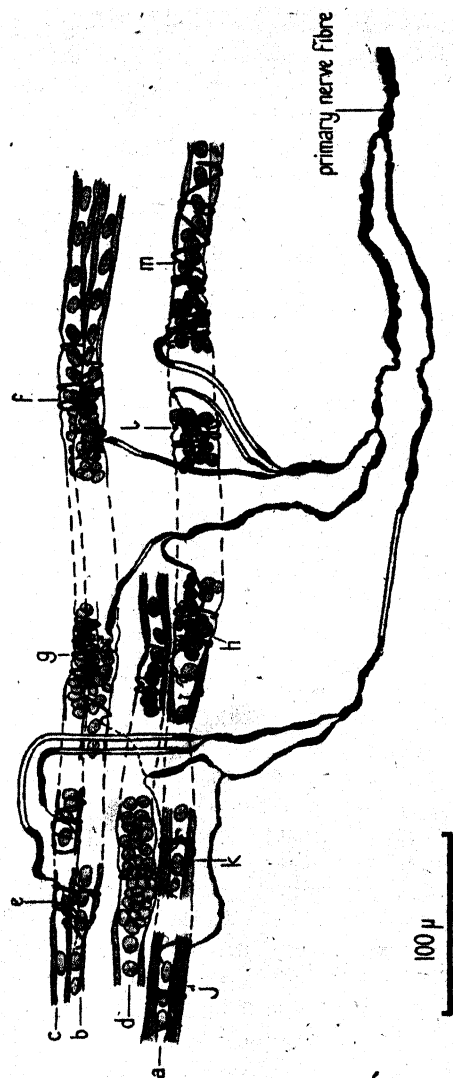
TEXT-FIG. 10. Schematic plan of the primary ending of the reconstructed rabbit's muscle-spindle. *a–d*, intrafusal muscle-fibres; *e–m*, portions of the ending referred to in text.

fibres with a total of twenty-two half-rings, three complete rings, and twelve spiral turns. This estimation is approximate in so far as it is an arbitrary matter as to how the more irregular portions of the ending may be classified.

In extensive types of primary ending (as shown in Text-fig. 7B and C) formed after an early subdivision of the primary fibre, that portion of the ending which occupies the myotube regions differs little from the rest. The ramifications tend to be composed of bands thinner than those surrounding the nuclear bags, and are not usually set so closely together, but their form is the same. For the most part they consist of half-rings set at right angles to the longitudinal axes of the muscle-fibres. More irregular clasping forms usually occur as the muscle-fibres pass out of the equatorial region and the limit of the area of termination is reached. Spirals occur but not in the loosely coiling diagonal form characteristic of the nuclear bag portion of the ending.

The finding of one sensory fibre dividing so that one branch forms a primary ending in a muscle-spindle, whilst the other terminates in an adjacent tendon-organ, has been reported to occur in various mammals by Kerschner (1888), Weiss and Dutil (1896a, b), Dogiel (1906), and Hines and Tower (1928). I have frequently found that one pole of a muscle-spindle (usually the distal) lies in association with a tendon organ. In four cases of such association the sensory fibres innervating the two proprioceptors were traced back as far as possible from their terminations. They were found to remain as independent fibres, and in two cases their approach was from different directions.

### The Secondary Ending

In the rabbit the form of the secondary ending differs only in minor details from that of the primary ending and the alternative term 'flower-spray' cannot be applied. So close is the resemblance that, in many cases, if the ramifications were to be judged solely by their form it would be difficult to decide whether they were derived from a secondary fibre or were part of an extensive primary ending such as is shown in Text-fig. 7B and C. The ending is, in fact, 'annulo-spiral' and consists for the most part of half-rings, rings, and spirals.

Subdivision of the secondary fibre was always found to occur after the penetration of the capsule, as Ruffini described. Some of the branches often trail for a considerable distance before terminating. The terminal ramifications, like those of the primary ending, may be confined to one muscle-fibre or spread over and interlock around two lying side by side. Each intrafusal fibre in the muscle-bundle usually bears a portion of the ending. The terminations are closely applied to the 'sarcoplasmatic membrane' and curl round the myotube regions of the muscle-fibres. In fixed preparations their coils form constrictions as in the case of the primary ending. The various systems of ramification are generally more dispersed than those of the primary sensory fibre and the flat ribbons of axoplasm are often narrower. In silver
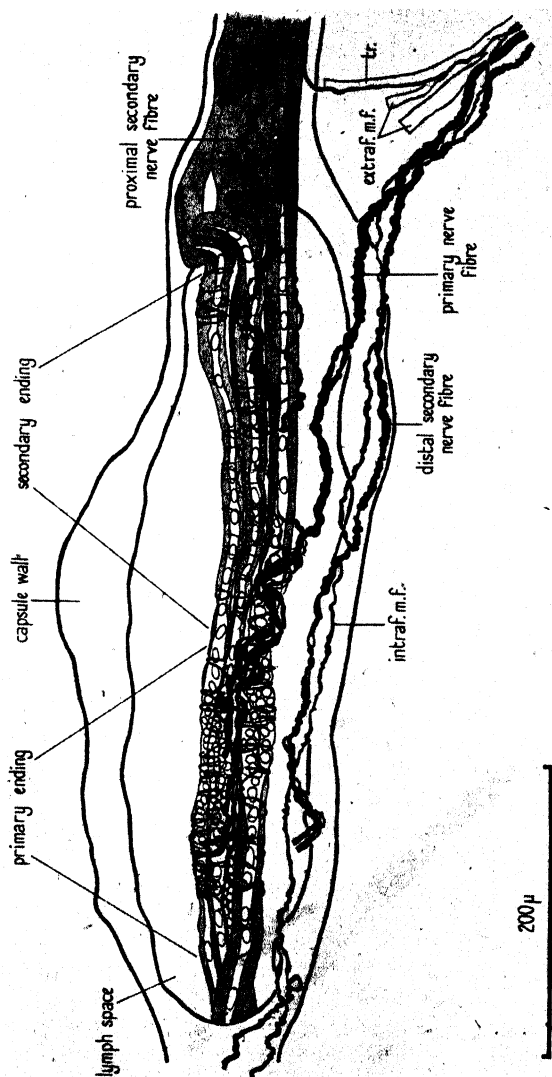
TEXT-FIG. 11. Equatorial region of the reconstructed rabbit's muscle-spindle. The distal secondary ending and such features as the axial sheath and trabecular connective tissue in the lymph space have been omitted to avoid confusion. *intraf.m.f.*, large intrafusal motor fibre innervating distal pole of spindle (see Text-figs. 3B and 4B, fibre *a*); *extraf.m.f.*, truncules carrying extrafusal motor fibres; *tr.*, truncule carrying proximal secondary nerve-fibre and motor fibres innervating proximal pole of spindle.

preparations they appear as fine tendrils rather than wide strands of neuro-fibrils, and wrap around the muscle-fibres so as to form a delicate tracery rather than a set of coarse bands.

Pl. II, fig. 5, shows the primary and secondary endings of an intermediate type of spindle from the vastus intermedius of a rabbit (gold chloride preparation). The secondary ramifications are seen to entwine the muscle-fibres in much the same way as those of the neighbouring primary ending. Whilst the primary ramifications are closely heaped together and occupy approximately 160 $\mu$ of the equatorial region, the secondary ending is more scattered and covers a length of approximately 480 $\mu$. The area covered is not always so extensive, but the terminal ramifications are invariably loosely rather than closely associated. Their 'annulo-spiral' nature is further illustrated in Pl. III, fig. 9, a photograph of the major portion of one of the secondary endings of a complex spindle impregnated with silver. The secondary terminations are seen to coil and spiral around the muscle-fibres in enlacing systems. Complete rings occur but they are uncommon; two are clearly seen in Pl. III, fig. 8, which illustrates part of a secondary ending from another complex spindle.

The similarity between the primary and secondary terminations is clearly demonstrated in Text-fig. 11, a drawing of the equatorial region of the complex spindle which was reconstructed as described on pp. 145, 146. The primary ending is shown together with the secondary ending which lay on its proximal side; the distal secondary ending has been omitted to avoid confusion. The secondary fibre enters the equatorial region and courses for some distance over the muscle-bundle before the first dichotomy occurs. Further branching eventually gives rise to a number of separate ramification systems which entwine the myotube regions of the four muscle-fibres. The ending has been schematically represented in Text-fig. 12, following the procedure that was adopted in the case of the primary ending (cf. Text-fig. 10). One of the terminal ramifications (*f*) could be said to be 'flower-spray' in form. It consists of a few fine tendrils which wrap around a muscle-fibre and at three places give rise to large leaf-like expansions of neurofibrils. The tendrils then disappear and break up into scattered clusters of granules. In some places these granules appear to be orientated with the cross-striations of the muscle-fibre, and it is difficult to decide whether they are part of the ending or a silver precipitation artifact. I have found similar ramification systems in a few other silver preparations of secondary endings. They have always occurred around a region of a muscle-fibre which, though equatorially placed, was not of the characteristic myotube form, for the central core of protoplasm was lacking and the central nuclei were few in number (as in Text-fig. 8, muscle-fibre *d*). It seems probable that such ramifications are the silver counterpart of similar irregular formations occasionally seen in gold chloride preparations (see *f.s* Pl. II, fig. 5). However, this is never the predominant mode of termination; the ramifications typically resemble the rest of those drawn in Text-fig. 12. The spiral complex (*s*) fortunately occurred intact in one
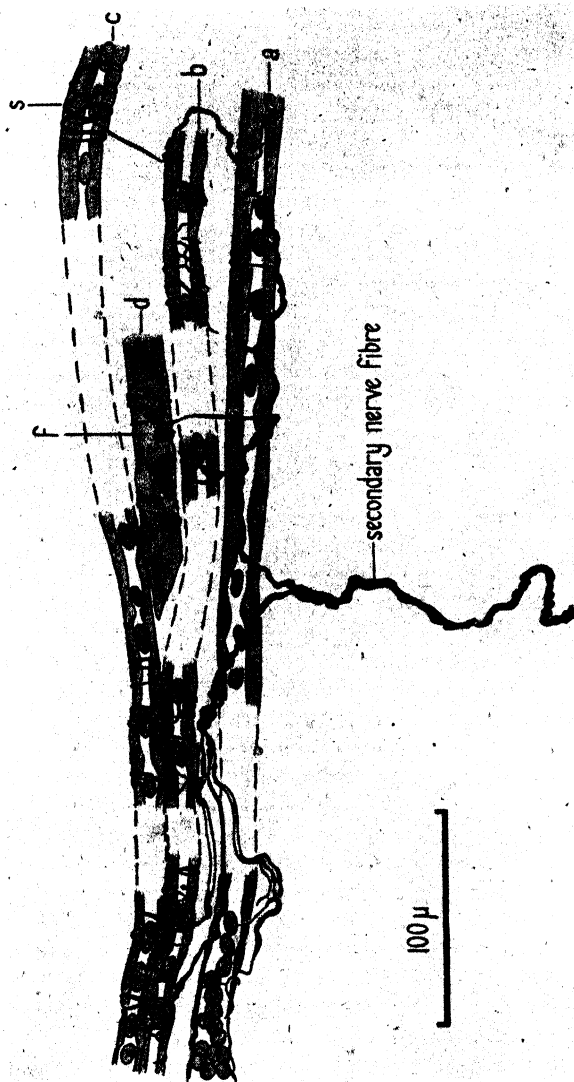
TEXT-FIG. 12.   Schematic plan of the proximal secondary ending of the reconstructed rabbit's muscle-spindle.  *a–d*, intrafusal muscle-fibres; *f*, *s*, portions of ending referred to in text.

section so that it was possible to demonstrate it photographically by optical sectioning (see Pl. III, fig. 7).

## The Diameters of the Sensory Fibres

The primary fibre runs within a wide (15–25 $\mu$) endoneurial sheath. It is the largest nerve-fibre innervating the spindle, with a total diameter falling within the range 8–12 $\mu$ (as estimated from measurements made on fresh, silver, and gold chloride material). Batten (1897, 1898) also found the primary fibres to be of these dimensions, his measurements being 8 and 10 $\mu$ in man, and 8 $\mu$ in the dog (Sihler preparations). The secondary fibres possess a smaller diameter which ranges between 6 and 9 $\mu$, and they run in a narrower endoneurial sheath. Typically the total diameter of a secondary fibre is equivalent to that of the axon diameter of the primary fibre innervating the same spindle.

The sensory fibres undergo a considerable increase in diameter as they approach their first subdivision; for example, one primary fibre increased from 10·8 to 12·6 $\mu$, another from 12·6 to 18 $\mu$. As Sherrington (1894) noted, the increase is due to a broadening of the axon whilst the myelin sheath becomes thinner. The same holds true for the sensory fibres innervating the tendon-organs, which otherwise are of the same dimensions as the primary fibres (8–12 $\mu$). Ruffini (1898) states that the primary fibre is larger than that innervating the tendon-organ, but he does not give any measurements. Sherrington (1894) gives 7–18 $\mu$ as the diameter range of sensory fibres innervating the muscle-spindle. However, when specifying the total diameters of two primary fibres in his description of the sensory innervation, he gives measurements of 8 $\mu$ and 12 $\mu$ (cat and monkey, gold chloride preparations). I have never observed sensory fibres approaching 18 $\mu$ calibre innervating either muscle-spindle or tendon-organ.

## The Sensory Innervation in the Cat

The primary ending in the cat, as seen in spindles from the quadriceps, has a strikingly regular appearance; the ramifications resemble a number of tightly coiled springs entwined around the nuclear bags of the muscle-fibres. Apart from their more regular disposition, however, the ramifications bear the same relationship to the muscle-fibres as in the rabbit. To either side of the central area of termination more irregular and widely spaced systems occur as Ruffini described (1898). The ending is usually of greater size than the primary of the rabbit, since in the rabbit the number of muscle-fibres in a spindle rarely exceeds four, whilst in the cat six or more are usually to be found. In simple spindles, as Ruffini observed, the ending is usually slightly less regular in form. The diameter of the primary fibre, as measured in gold chloride preparations, most often lies between 8 and 12 $\mu$ as in the rabbit, though I have found one measuring 14·8 $\mu$.

The secondary ending, on the other hand, is more irregular than its counterpart in the rabbit. The muscle-fibres are entwined with spirals, half-rings, and clasping systems which tend to form a background for the 'flower-spray' type of ramification. Some of these 'sprays' spread over the surface of the

muscle-bundle and are not related to particular muscle-fibres. However, the ending is never wholly 'flower-spray' and it is possible that Ruffini exaggerated this characteristic in his efforts to establish the existence of two types of termination. The ending most often resembles Ruffini's drawing of an irregular type of primary ending (1898, fig. 3), where a number of loops and spirals are shown amongst tree-like ramifications. Tello (1922) evidently found the ending to be more of this form in material from 30-day-old cat embryos; his term 'claw-like ending' (*krallenförmige Endigung*) would be more expressive of the general mode of termination if a descriptive term were required. The diameter of the fibre forming the ending varies, as in the rabbit, between 6 and 9 $\mu$ (measurements made on gold chloride material).

Pl. II, fig. 1, illustrates the general appearance of the two types of ending in the cat as they occurred in a complex spindle from m. vastus medialis (gold chloride preparation). In Pl. II, figs. 2, 3, the primary and secondary endings are shown at a greater magnification. Spindles with one primary and one secondary ending did not occur in the limited number of spindles examined, in contrast to the distribution in the rabbit's quadriceps and interossei where this type appears to be the most numerous. Complex spindles (with one primary and two secondary endings) were the most frequent, and this agrees with Ruffini's finding. In eight spindles from mm. vastus medialis and subcrureus, five were complex and three simple.

### The Use of the Terms 'Annulo-spiral' and 'Flower-spray'

When it is desired to distinguish between the two types of sensory ending present in muscle-spindles the terms 'annulo-spiral' and 'flower-spray' are commonly used in preference to Ruffini's alternatives 'primary' and 'secondary'. However, in the rabbit the descriptive terms are not applicable, since both endings have an 'annulo-spiral' form. Moreover, it is evident from the literature that either termination may exist in the 'annulo-spiral' or 'flower-spray' form according to the animal in which it occurs. In the frog there appears to be only one sensory ending present, and its association with the nuclear bags of the intrafusal muscle-fibres suggests that it corresponds to the primary ending in mammals. According to the observations of Cajal (1888), Dogiel (1890), and Huber and de Witt (1898) on frog muscle-spindles, this ending consists of a number of varicose threads running parallel to the muscle-fibres. As judged from their figures, and the few gold chloride preparations I have made of frog spindles, the general form of this primary ending is more 'flower-spray' than 'annulo-spiral'. In reptiles, birds, and mammals the primary ending is 'annulo-spiral' though its regularity varies. In mammals its regularity in the dog (Batten, 1898) and mouse (Kerschner, 1893) approaches that found in the cat, whilst in man (Kerschner, 1893; Ruffini, 1893), rat (Kerschner, 1893; Huber and de Witt, 1898), and guinea-pig (Huber and de Witt, 1898) the spiral pattern is less evident and the ending resembles the form occurring in the rabbit.

Secondary endings of the 'flower-spray' form as found in the cat have been

observed in the hedgehog (Garven, 1925), the pig (Cuajunco, 1927), and man (Ruffini, 1893). Tello (1922) described its form as ‚'claw-like' in six-month human foetal material. Denny-Brown (1928a) found secondary endings to occur in complex spindles from the extrinsic eye-muscles of sheep, and maintained that they were identical with those found in similar spindles from the cat's gastrocnemius. Cilimbaris (1910), studying the same material, figured endings which were apparently secondary and of the 'flower-spray' type, though he made no attempt to correlate his observations with Ruffini's. The ending has not been previously explicitly recognized in its 'annulo-spiral' form, although the figures of Huber and de Witt (1898, fig. 33) and Dogiel (1902, figs. 14, II and v) clearly show spindles of the rabbit with primary and secondary endings both of the 'annulo-spiral' type. Faced by one extensive system of spirals rather than by two distinct types of termination, Dogiel concluded that Ruffini's descriptive terms applied to the various regular (annulo-spiral) and irregular (flower-spray) *portions* of the endings and considered such a classification superfluous. Similarly Huber and de Witt supposed that Ruffini's 'flower-spray' ending was merely the more irregular terminal portion of a spiral ramification. It seems highly probable that the spindles described by Huber and de Witt as 'compound', with two or three 'areas of nerve distribution', were spindles with a primary ending accompanied by one or two secondary endings. They found such spindles in the tortoise, dove, and various mammals. They illustrate a 'compound' spindle from the intrinsic plantar muscles of the dog (1898, fig. 39) which is without doubt of the complex type that occurs in the rabbit, with a secondary ending of 'annulo-spiral' form lying on each side of the primary ending.

In view of the varying form of the two types of sensory termination the terms 'primary' and 'secondary' would seem preferable. These terms adequately express the facts that the primary ending is always present whilst the secondary ending only occurs in the intermediate and complex type of spindle; that the primary fibre is larger than the secondary; and that during development, according to Tello (1922), the primary fibre is the first to reach the end-organ whilst the secondary fibres arrive later together with the motor fibres.

### Other Nerve-fibres innervating the Muscle-spindle

The muscle-spindle receives a rich vascular supply, and fine nerve-fibres are commonly seen accompanying the blood-vessels. In silver preparations they run a characteristically sinuous course and measure approximately 0·5 $\mu$ in diameter. There is no increase in diameter before branching, nor any constriction at the point of division. The divisions are dichotomous and the branches diverge widely apart. These fibres have previously been noted by Huber and de Witt (1898), Dogiel (1902), and Tello (1922), and were considered by these authors to be sympathetic. This was later proved correct by Hinsey (1927), who found that such fibres were the only ones to remain intact after a degeneration of the somatic innervation of the spindle, and by

Hines and Tower (1928) who found them to be the only spindle nerve-fibres which degenerated in sympathectomized material.

There are other fibres, finer than these, which appear to take part in the innervation of the spindle itself. Perroncito (1902) first noted their existence in muscle-spindles of the lizard. He observed two or three very thin fibres which approached the spindle together with the primary fibre and apparently ran within its endoneurial sheath. On reaching the equatorial region they ramified and spread out to form an anastomosing network within the walls of the capsule. Tello (1922) observed similar fibres innervating spindles of the cat and man in the later stages of development. They bore no relation to the
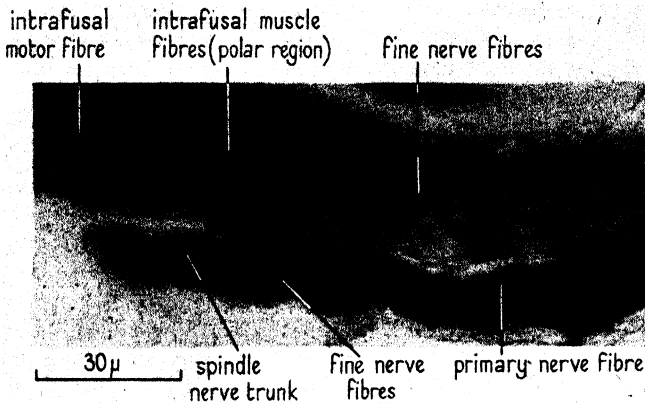


TEXT-FIG. 13. Part of a polar region of a rabbit's muscle-spindle with portion of spindle nerve-trunk alongside. Very fine fibres accompany the primary nerve-fibre in the nerve-trunk and also course over the polar region. M. vastus intermedius, L.S. 25 μ, de Castro silver method.

blood-vessels but ran chiefly within the layers of the capsule so as to encircle the equatorial region. Sometimes the fibres appeared to leave the spindle without terminating; alternatively they formed endings on the intrafusal muscle-fibres, though the mode of termination was obscure. Garven (1925, m. panniculus carnosus of hedgehog) describes fine unmyelinated fibres entering the spindle with the other nerve-fibres and forming a complex network of ramifications inside the capsule. He failed to find them ending upon the muscle-fibres. On the other hand, Creed et al. (1932) state that unmyelinated fibres, entering the spindle with the primary fibre, frequently end 'as minute rings or plates epilemmally placed among the nuclei of the sheath of the intrafusal muscle-fibre, often in the region of the annulo-spiral ending'. Cuajunco (1940, human foetal material) maintains that branches of unmyelinated fibres actually join the ramifications of the sensory endings. Since other branches appeared to innervate the walls of blood-vessels he concluded that the fibres were of sympathetic origin.

In the rabbit spindles examined these fine fibres were not a constant occurrence and could only be detected in silver preparations (see Text-fig. 13). They are so small (less than $0.5\ \mu$) that it is impossible to be certain of the presence or absence of a myelin sheath. Sometimes one such fibre penetrates the capsule with a sensory fibre, takes a meandering circling course within the capsule walls, and proceeds into one of the polar regions. Here it divides extensively to form a fine anastomosing network. Alternatively, a small bundle of these fibres may enter the spindle at one pole together with other spindle nerve-fibres. Some of them divide so as to form a network over part of this polar region, whilst others, dividing *en route*, wind their way through the walls of the capsule and form a network in the other polar region. I have been unable to determine whether this network is confined to the connective tissue sheath surrounding the intrafusal muscle-bundle, or whether it is in contact with the muscle-fibres themselves. In the spindles examined the fibres have never been observed to end amongst the sensory terminations.

The fact that these fibres frequently approach a spindle in the form of a bundle, their course and distribution within the spindle, and their occurrence in one spindle but absence in another nearby in the same preparation, leaves no doubt that these fibres are nervous and not artifacts. Similar fibres have been described as taking part in the innervation of tendon-organs by Ruffini (1897, 'concomitant fibres') and Tello (1922, 'accessory fibres'). Dogiel (1902) also observed thin myelinated fibres forming a fine network within small types of Pacinian corpuscles. I have seen such a corpuscle, innervated as he describes, lying under an aponeurosis of m. vastus medialis of a lamb. The nature and function of these fibres is at present unknown. It is possible that they are somatic sensory fibres subserving the sensation of pain.

### DISCUSSION

It has been shown by Sherrington (1894) and Eccles and Sherrington (1930) that afferent fibres $12$–$20\ \mu$ in diameter are present in considerable numbers in muscular nerve-trunks. On histological grounds there is no alternative but to suppose, as Sherrington (1894) suggested, that the majority of these large fibres terminate in muscle-spindles and tendon-organs, while a certain number are presumably accounted for by Pacinian corpuscles. However, some reconciliation must be made between the fact that when the large primary fibres innervating the muscle-spindle and the fibres innervating the tendon-organs are measured in the neighbourhood of these end-organs, their diameter lies between 8 and $12\ \mu$ and rarely exceeds $12\ \mu$, while fibres of this size are poorly represented in the muscular nerve-trunk. It is possible that larger afferent fibres subdivide into fibres of these dimensions, though the data of Eccles and Sherrington suggest that very little subdivision of afferent fibres above $10\ \mu$ occurs as the nerve-trunk approaches the muscle. Intramuscular subdivision has been observed by Golgi (1880), Cattaneo (1888), and Dogiel (1906) to occur in the case of the tendon-organ afferents, one fibre branching so as to innervate several end-organs, and a similar condition has

often been described for the innervation of Pacinian corpuscles, but the sensory fibres innervating the muscle-spindle show no sign of being derived by branching from a parent fibre when traced back a few millimetres from the spindle. The discrepancy in the diameter measurements might be due to the diameter of the fibres progressively diminishing in their course from dorsal root to end-organ. The observations of Sherrington (1894) on osmic material suggest that such diminution may occur; an examination of several post-thoracic dorsal roots revealed a number of fibres 23–5 $\mu$ in diameter, whilst in the muscular nerve-trunks the largest fibre found was 20·5 $\mu$ and fibres of 20 $\mu$ were rare, and in intramuscular nerve-trunks the fibres were rarely 'quite as large as the largest afferent fibres seen in the parent nerve-trunks traceable into the muscle by naked eye dissection' (p. 235).

Whatever the explanation of this discrepancy, the fast conduction rate of the afferent impulses initiated when a muscle is stretched supports the conclusion that the 12–20 $\mu$ group of afferent fibres in muscular nerve-trunks is proprioceptive. Recently Lloyd (1943) has applied brief stretch stimuli to the cat's gastrocnemius and recorded the resultant afferent discharge at several points along the nerve and dorsal root between the muscle and spinal cord. He showed that the afferent response was conducted at an average maximum velocity of 116 m.p.s. According to the size: velocity ratio for the cat (Hursh 1939) fibres with this conduction rate have a diameter of 18 $\mu$. It is therefore evident that stretch receptors in the muscle are innervated by the 12–20 $\mu$ group of afferent fibres. It is impossible to determine histologically which fibres within this range ultimately become the primary (8–12 $\mu$) and secondary (6–9 $\mu$) afferents which enter the muscle-spindles, and the afferents (8–12 $\mu$) which innervate the tendon-organs. The individual conduction rates of these fibres are not known, though in Matthews's (1933) single fibre preparations the time relations of the impulses recorded from the secondary endings of the muscle-spindle (A1 receptors) showed that they travelled in smaller fibres than those from the primary spindle endings and tendon-organs (A2 and B receptors). This suggests, together with Lloyd's figure of 116 m.p.s., that the larger fibres within the 12–20 $\mu$ range innervate the tendon-organs and terminate as primary endings in the muscle-spindles, and that the secondary spindle afferents are to be identified amongst the smaller fibres in this group.

Matthews (1933) concluded from his experiments that both the primary and secondary endings of the muscle-spindle responded when the muscle was stretched, but that only the primary ending was affected during contraction caused by supramaximal motor stimulation. He assumed that supramaximal stimuli excited the small motor fibres innervating the spindle and caused the intrafusal muscle-fibres to contract. The different behaviour of the two types of ending under these conditions was accounted for by supposing that whilst the non-contractile nuclear bags would become extended and so distort the primary endings during the contraction of the intrafusal muscle-fibres, the myotube regions would shorten and so decrease the distortion of the secondary endings. Impulses would therefore be initiated in one but not the other. The different

location of the two types of ending may well result in their behaving differently during intrafusal contraction, but it must be noted that the strongest motor stimulus used by Matthews was 'at least' 30 per cent. supra-maximal as judged by the height of the contraction attained by the muscle, and in view of Leksell's work (1945) it is clear that such stimuli would fail to excite the greater proportion of the small ('gamma') efferent fibres innervating the spindle. Leksell showed that a muscle attained its maximum contraction tension at a stimulus strength 2·5 times the threshold of the largest fibres in the 'alpha' group, and this agreed with the figure previously obtained by O'Leary, Bishop, and Heinbecker (1934). The whole 'alpha' group was activated when the stimulus was on an average 3·1 times that of the threshold of its most excitable fibres. The threshold of the 'gamma' efferent fibres was on an average 3·9 times the 'alpha' threshold, and a further 12-fold increase of stimulus strength was required to excite all the fibres within this group. Hence a motor stimulus approximately 30 per cent. supramaximal, such as used by Matthews, would only be sufficient to excite the larger motor fibres innervating the spindle. To ensure the maximum contraction of the intra-fusal muscle-fibres a stimulus would have to be employed that was more than 500 per cent. supramaximal as judged by the height of the muscle contraction. It remains for future experiment to show whether the secondary endings would respond under these conditions.

At present the manner in which the muscle-fibres of the spindle contract is a matter for speculation, and nothing is known of the conditions of central excitation necessary for this to occur. It has been shown in this paper that in all probability each member of the muscle-bundle is a double contractile unit, the two polar portions being separated by a non-contractile nuclear bag, and each end bearing one (sometimes two) motor end-plates. It is possible that the intrafusal muscle-fibre should on the contrary be regarded as a single contractile unit, the effect of its multiple motor innervation being to ensure the simultaneous contraction of the two poles in much the same way that the possession of multiple end-plates appears to ensure an almost simultaneous contraction of the entire length of the extrafusal muscle-fibre in the frog's sartorius (see Katz and Kuffler, 1941). However, the fact that the two poles are separated by a region full of nuclei and devoid of cross-striations, and are often innervated by motor fibres differing considerably in diameter, would appear to weigh against this view. In those spindles where one polar region is innervated by small ('gamma') motor fibres and the other by a relatively large ('alpha') motor fibre, it seems probable that the contraction of the two poles will not be simultaneous. If each pole is innervated by large and small motor fibres their differing rates of conduction may result in the individual polar units contracting separately. Since each sensory fibre entering the spindle invariably ramifies so as to form endings around each muscle-fibre, the question arises as to what the nature of the impulses discharged from the entire termination would be during such progressive contraction. It is interesting to note in this connexion that in some of his recordings from primary endings

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during contraction Matthews found that the change-over from silence to response was gradual, the rate of discharge increasing as the motor stimulus was increased. He suggested that this might be due to the muscle-fibres of the spindle contracting separately.

Leksell found that the conduction rate of motor fibres in the small 'gamma' group was 20–38 per cent. that of the largest 'alpha' motor fibres. Hence it is possible that the intrafusal muscle-fibres innervated by this group will still be in a state of contraction when the ordinary muscle-fibres are relaxing. It has been shown that the extreme ends of the muscle-fibres of one or both poles of a spindle often taper off and fuse with the endomysium of ordinary muscle-fibres. During the relaxation of the muscle, therefore, the elongation of the extrafusal muscle-fibres by their antagonists may apply a stretch stimulus to the contracting muscle-fibres of the spindle. Maybe it is only under these conditions that impulses are discharged by the secondary endings in the course of contraction. However, these matters can only be elucidated by studying the behaviour of the sensory endings in single fibre preparations at all gradations of motor stimulation.

The form and disposition of the sensory endings of the spindle suggest that they are stimulated by mechanical distortion. During stretch, as Matthews (1933) remarks, 'it is difficult to see how tension could stimulate except by the distortion it produces.' During contraction it would seem unnecessary to postulate any other mode of stimulation such as by the action potential of the intrafusal muscle-fibres, or by pressure exerted on the endings by the contracting extrafusal muscle-fibres via capsule and lymph space. It has been suggested by Hinsey (1927) that when the intrafusal muscle-fibres contract the nerve-endings may be stimulated by being pressed against the axial sheath which overlies them. The 'flower-spray' type of ramification which spreads over the surface of several muscle-fibres would appear to be well suited to respond to this form of stimulation, but the annular and clasping forms of ending are more probably primarily adapted for stimulation by a change in form of the muscle-fibres they entwine. At present it is difficult to ascribe any functional significance to the regularity or irregularity of a sensory spindle ending. The primary ending of the cat (Matthews, 1933) has been found to respond during stretch and contraction in a similar way to the corresponding ending in the frog (Matthews, 1931b), yet these endings are markedly dissimilar in form (see p. 172). In both animals the ending is associated with the nuclear bags of the intrafusal muscle-fibres which suggests that it is the position of the endings within the equatorial region rather than their form that is the important factor affecting the response. Mechanical distortion would also appear to serve as the mode of stimulation for the tendon-organ endings. This may be partly provided by stretch (Eccles, personal communication to Matthews, 1933), but the distribution of these end-organs in the deep rather than the superficial laminae of tendons (Golgi, 1880), and their occurrence within the muscle in the neighbourhood of aponeuroses and intramuscular tendons, suggest that distortion by compression may also provide a mode of stimulation.

It has long been apparent that among the afferent fibres proceeding from a mammalian extensor muscle such as the quadriceps there are some which reflexly excite its contraction and others which reflexly inhibit it. Though there is every reason to suppose that the afferent fibres concerned in this excitation and inhibition are those innervating the muscle-spindles and tendon-organs, the identification of the sensory endings which fulfil these opposing functions still awaits conclusive demonstration. In attempting to account for the cessation of action currents in a muscle ('silent period') which occurs during its brief reflex contraction in response to stretch (as in knee- and ankle-jerks), Fulton and Pi-Suñer (1928) and Denny-Brown (1928a, b) were led to propose conflicting theories with regard to the reflex effects of the afferent impulses discharged by these receptors. Fulton and Pi-Suñer concluded that the impulses set up by the muscle-spindle were excitatory and that the silent period was due to the fact that these end-organs ceased to discharge when unloaded by extrafusal contraction ('in series' behaviour). They suggested that impulses initiated by the tendon-organs, which lay 'in parallel' with the contractile elements, were probably inhibitory. Denny-Brown, on the other hand, believed that the reflex effect of the tendon-organ discharge was excitatory, and supposed that efferent activity during the silent period was initially prevented by a refractory state of the motor neurones and later prevented by an inhibition arising from the sensory endings of the muscle-spindle. When Denny-Brown advanced this view the presence of an afferent discharge from the spindle during stretch was open to doubt and he formulated his hypothesis on the assumption that the sensory endings of the spindle discharged only during contraction. However, Matthews (1931a, b) was able to provide conclusive evidence that impulses were set up by the spindle both during the stretch and the contraction of muscle, and the behaviour of his single fibre preparations (1933) conformed with that proposed by the 'in series, in parallel' hypothesis. Matthews supposed that the silent period was due partly to an inhibitory discharge from the tendon-organs and partly to the absence of an excitatory discharge from the muscle-spindles. The silent period was known to be most pronounced during the contraction of muscle at low initial tensions, and Matthews's recordings showed that the tendon-organ was the only receptor which discharged during contraction under these conditions. Therefore if the latter part of the silent period was inhibitory in origin as Denny-Brown had maintained, the tendon organ alone could be held responsible for it. Matthews believed that the contraction of muscle in the stretch reflex was excited by the muscle-spindles, and suggested that their secondary endings should be regarded as those which initiated the excitatory impulses on account of their very low threshold of response to stretch. He was undecided as to the reflex nature of the discharge from the primary ending of the spindle, but inclined to regard it as inhibitory.

The spindle is now generally assumed to be the receptor which excites the stretch reflex and in this connexion is often simply referred to as the 'stretch afferent'. However, much of the evidence advanced in support of this view

is either invalid or inconclusive, and its acceptance ultimately depends upon Matthews's finding of a considerable difference in threshold between the spindle and tendon-organ in response to stretch. In his preparations a tension of 1–2 gm. was sufficient to evoke a rhythmic discharge from the secondary ending of the spindle and the primary ending discharged at tensions of 5–10 gm.; on the other hand, in the case of the tendon-organ, a tension of 20–200 gm., or in some preparations even 700 gm., was required to initiate a discharge. The low threshold of the receptor exciting the stretch reflex is indicated by the fact that stretches of a fraction of a millimetre are sufficient to elicit the contraction (see, for example, Liddell and Sherrington, 1924; Denny-Brown and Liddell, 1927; Lloyd, 1943). A correlation of this characteristic of the reflex with Matthews's threshold data therefore suggests that it is the spindle rather than the tendon-organ which excites the response. If this is so it is clear that the large primary fibre innervating the spindle should be identified as the 'stretch afferent' rather than the smaller secondary fibre specified by Matthews, for the brief latency of the reflex and rapid conduction rate of the afferent discharge exciting it (Lloyd, 1943) indicate that sensory fibres of the largest diameter are employed. The functional significance of the secondary endings, their occurrence in some spindles in mammalian muscle but not others, and their complete absence from amphibian spindles are matters to which at present there is no answer. The reflex nature of the afferent discharge from the spindle during intrafusal contraction is equally obscure. Does the spindle excite the contraction of its own muscle-fibres and in so doing initiate a further excitatory discharge from its sensory endings?

The low threshold of the spindle in response to stretch suggests that it may well be the receptor which excites the stretch reflex, but the other evidence which has been advanced in support of this view does little to strengthen it. For example, it has been argued (Fulton, 1926; Fulton and Pi-Suñer, 1928) that since the stretch reflex of a muscle is unimpaired when its tendon is cut away or anaesthetized, the tendon-organ can be excluded as being the proprioceptor which bears the excitatory afferent. The experiments of Morson and Phillips (1937) were also based on the assumption that tendonectomy ensures the removal of the majority of tendon organs. However, the tendon-organs of a muscle are found wherever the muscle and tendon fibres composing it are united. Hence they occur not only at the musculo-tendinous junctions at either end of a muscle, but also in the places where these junctions are intramuscular and under flattened expansions of tendon (aponeuroses) covering the fleshy part of a muscle. Moreover, they frequently occur in association with muscle-spindles. Therefore, as Hinsey (1927) and Denny-Brown (1928a) have indicated, it is impossible to achieve any mechanical separation of the two types of proprioceptor which would ensure a significant decrease in the number of tendon-organs relative to muscle-spindles. The fact that the stretch reflex is strictly confined to the portion of the muscle stretched is also cited as circumstantial evidence in favour of regarding the muscle-spindle as the end-organ which excites the response. However, the distribu-

tion of muscle-spindles and tendon-organs does not warrant this deduction; both types of proprioceptor are usually to be found in any small block of muscle taken from members of the quadriceps of the cat and rabbit.

Finally, it is urged that the muscle-spindle should be regarded as the receptor which excites the stretch reflex, since it is lacking in those muscles not subject to gravitational stretch (Fulton, 1946), and in these muscles it is commonly supposed that the stretch reflex is absent. This implies that the distribution of muscle-spindles in the body is confined to anti-gravity muscles, but it is evident from the literature (see especially Gregor, 1904) that this is not the case. Muscle-spindles occur in the diaphragm (Dogiel, 1906), in the extrinsic muscles of the larynx (Kölliker, 1889; Forster, 1894; Amersbach, 1911), and tongue (Forster, 1894; Langworthy, 1924), and are particularly abundant in muscles of fine adjustment, for example, of the hand (see, for example, Voss, 1937). Cilimbaris (1910) found a spindle in m. tensor tympani of the sheep. None of these muscles could be said to fulfil an anti-gravity function. Of the muscles in which it has been alleged that spindles are absent (intrinsic laryngeal and lingual muscles, genital, facial, and extrinsic eye-muscles) evidence about their stretch-reflex response is known only for the extrinsic muscles of the eye. This reflex could not be demonstrated in these muscles by Irvine and Ludvigh (1936) in man, nor by McCouch and Adler (1932) or McIntyre (1941) in the cat. McIntyre, moreover, failed to detect the presence of any sensory discharge during the stretch and contraction of these muscles in the cat. The reason for this is at present obscure, but these observations cannot be regarded as evidence for supposing that the muscle-spindle excites the stretch reflex since the assertion that spindles are lacking in the extrinsic eye-muscles is incorrect. They have been demonstrated to occur in their usual encapsulated multifibral form in human eye-muscles (Cooper and Daniel, 1948) and in the eye-muscles of the sheep and various mammals by Cilimbaris (1910), while they appear to be present in the unencapsulated monofibral form in the eye-muscles of the cat (Denny-Brown, 1928*a*; Pallot, 1934).

Thus the view that the 'stretch afferent' is borne by the muscle-spindle is seen to rest largely upon equivocal evidence and to receive convincing support only from the fact that it is the most probable conclusion to be drawn from Matthews's threshold data. On the other hand, it may equally well be said that there is little evidence to suggest that the excitatory receptor should on the contrary be identified as the tendon-organ. At present the only indication that this alternative view may be correct is provided by experiments in which the recovery of the stretch reflex has been observed after nerve regeneration. Recoveries have been reported by de Rezende (1942) and Davis *et al.* (1945) after nerve-grafting, and by Barker and Young (1947) after nerve-suture. In the latter investigation the reflex (knee-jerk) was found to recover to the extent of developing over half its pre-operative tension in some cases. After the complete division of a nerve one would suppose that a tendon-organ, innervated by one sensory fibre, would stand a greater chance of becoming successfully reinnervated than a muscle-spindle, which receives both a motor and a

sensory innervation. Considerable recoveries of the stretch reflex after such operations therefore suggest that the afferent fibre which excites the response belongs to the tendon-organ rather than the muscle-spindle. It remains to be seen whether a full investigation of the muscles reinnervated in Barker and Young's experiments will justify these conclusions. A preliminary examination of spindles reinnervated after nerve-suture suggests that it is very doubtful whether the motor and sensory innervation is ever fully restored.

It seems probable that all myotatic phenomena are the outcome of a fluctuating balance between two types of afferent discharge arising from muscle which have opposing reflex effects, one exciting and the other inhibiting contraction somewhat on the lines suggested by Denny-Brown (1928a, b) and Creed et al. (1932). Judging from the balance of present evidence it seems probable that the afferent discharge from the spindle is excitatory and that from the tendon-organ inhibitory to the motor neurones of the same muscle. However, the specific reflex functions of the sensory fibres innervating these receptors, and the manner in which the impulses they carry interact during the stretch and contraction of muscle, clearly require further elucidation.

## Summary

A study of the morphology and innervation of muscle-spindles from the quadriceps of the rabbit and cat has shown that:

1. The intrafusal muscle-fibres do not subdivide in their course through the spindle, as is maintained in some descriptions, but retain their individuality from pole to pole.

2. There is no constant feature which is characteristic of one pole of a spindle and not the other. A distinction can be made between the proximal and distal ends only when it is possible to orientate the spindle according to the proximal and distal ends of the muscle. The extreme ends of the spindle are attached indifferently to extrafusal endomysium, tendon, or perimysial connective tissue.

3. In the equatorial region each muscle-fibre of the spindle contains a dense aggregation of spherical central nuclei ('nuclear bag'). On either side of this aggregation oval nuclei are disposed in the form of a chain within a central core of protoplasm ('myotube region'). The nuclear bag is devoid of cross-striations and presumably non-contractile. The two polar portions of the muscle-fibre on either side of the bag are striated and each receives a motor innervation; hence they are presumed to function as independent contractile units.

4. The number of end-plates possessed by a spindle is approximately double its number of intrafusal muscle-fibres, with half the total number of end-plates situated at each pole. The ratio is rarely exact, since one polar half of an intrafusal fibre frequently bears two end-plates; these are innervated by nerve-fibres which retain their individuality as far as they can be traced back from the spindle. Both small nerve-fibres ($3-4$ $\mu$ in gold chloride preparations) and relatively large nerve-fibres ($6-7$ $\mu$ in gold chloride preparations) take part in the motor innervation of muscle-spindles, as was deduced on physiological grounds by Leksell (1945).

5. An analysis of the sensory innervation has confirmed many of Ruffini's (1898) observations. Primary or 'annulo-spiral' and secondary or 'flower-spray' endings occur and they are innervated by independent nerve-fibres; it is suggested that Ruffini's terms 'primary' and 'secondary' be adopted since the descriptive terms cannot always be applied. In the rabbit the secondary ending is 'annulo-spiral' in form and differs little from the primary ending; in the cat it is more irregular and could be termed 'flower-spray'. The primary ending is always present and is associated with the nuclear bags of the intrafusal muscle-fibres; in some instances its ramifications are more extensive and also entwine the myotube regions. The primary ending may be the only sensory termination present, or it may be accompanied by one or by two secondary endings. These are borne by the myotube regions of the muscle-fibres. In the rabbit's quadriceps and interossei, spindles with one primary and one secondary ending were the most frequent in the samples taken; in the cat's quadriceps spindles with one primary and two secondary endings were the most numerous. Both the primary and secondary nerve-fibres invariably ramify so as to innervate each intrafusal fibre in the muscle-bundle. The two sensory terminations are often closely intercalated but do not overlap with one another to any great extent. As estimated from measurements made on fresh, silver, and gold chloride preparations the total diameter of the primary fibre lies between 8 and 12 $\mu$, that of the secondary fibre between 6 and 9 $\mu$.

6. Apart from small sympathetic fibres innervating the vascular supply of the spindle, other finer fibres may occasionally be seen ramifying within the walls of the capsule and over the polar regions. It is possible that they are somatic sensory fibres subserving the sensation of pain.

7. The nature of the reflex effects of the afferent impulses discharged by the muscle-spindle and tendon-organ is considered, and it is concluded that the balance of evidence indicates that the afferent discharge from the spindle is excitatory and that from the tendon-organ inhibitory to the motor neurones of the same muscle. However, the identification of the spindle as the receptor which excites the stretch reflex is found to rest largely upon equivocal evidence, its acceptance depending ultimately upon Matthews's finding (1933) of a considerable difference in threshold between the spindle and tendon-organ in response to stretch. It is suggested that the large primary fibre innervating the spindle should be identified as the 'stretch afferent' rather than the smaller secondary fibre specified by Matthews, for the rapid con-

duction rate of the afferent discharge exciting the stretch reflex (Lloyd, 1943) indicates that sensory fibres of the largest diameter are employed. The functional significance of the secondary fibres is obscure and the specific reflex functions of the sensory fibres innervating both the spindle and the tendon organ clearly require further elucidation.

## REFERENCES

AGDUHR, E., 1919. Anat. Anz., **52**, 273.

—— 1939. Upsala Läkarefören. Förhandl., **45**, 401.

AMERSBACH, K., 1911. Beitrage z. Path. Anat., **51**, 56.

BARKER, D., and YOUNG, J. Z., 1947. Lancet, **1**, no. 19, 704.

BATTEN, F. E., 1897. Brain, **20**, 138.

—— 1898. Ibid., **21**, 388.

BAUM, J., 1900. Anat. Hefte, Abt. 1, **13**, Heft 42, 251.

BENSLEY, B. A., 1938. *Practical Anatomy of the Rabbit*, 6th ed. Revised by E. H. Craigie. Toronto.

BOEKE, J., 1916. Verh. Akad. Wet. Amst., **18**, 1.

—— 1927. Zeit. f. mikr.-anat. Forsch., **8**, 561.

BREMER, L., 1883. Arch. mikr. Anat., **22**, 318.

CAJAL, S. R., 1888. Rev. trim. Histol. nom. y patol., **1**, fasc. 1.

DE CASTRO, F., 1925. Trav. lab. rech. biol. univ. Madrid, **23**, 427.

CATTANEO, A., 1888. Arch. Ital. de Biol., **10**, 337.

CILIMBARIS, P. A., 1910. Arch. mikr. Anat., **75**, 692.

COOPER, S., and DANIEL, P., 1948. Brain (to be published).

CREED, R. S., DENNY-BROWN, D., ECCLES, J. C., LIDDELL, E. G. T., and SHERRINGTON, C. S., 1932. *Reflex Activity of the Spinal Cord.* Oxford (Clarendon Press).

CUAJUNCO, F., 1927. Carnegie Inst. Wash. Pub., no. 380, **19**, 45.

—— 1932. J. Comp. Neurol., **54**, 205.

—— 1940. Carnegie Inst. Wash. Pub., no. 518, **28**, 97.

DAVIS, L., PERRET, G., HILLER, F., and CARROLL, W., 1945. Surg. Gynec. Obstet., **80**, 35.

DENNY-BROWN, D. E., 1928a. *On the Essential Mechanism of Mammalian Posture.* D.Phil. thesis, Oxford University.

—— 1928b. Proc. Roy. Soc. B, **103**, 321.

—— and LIDDELL, E. G. T., 1927. J. Physiol., **63**, 70.

DICKSON, L. M., 1940. J. Anat., **74**, 268.

DOGIEL, A. S., 1890. Arch. mikr. Anat., **35**, 305.

—— 1902. Ibid., **59**, 1.

—— 1906. Ibid., **67**, 638.

ECCLES, J. C., and SHERRINGTON, C. S., 1930. Proc. Roy. Soc. B, **106**, 326.

FORSTER, L., 1894. Virchows Arch., **137**, 121.

FULTON, J. F., 1926. *Muscular Contraction and the Reflex Control of Movement.* Baltimore (Williams & Wilkins Co.).

—— 1946. Howell's Textbook of Physiology, 15th ed. Philadelphia and London (W. B. Saunders Co.).

—— and PI-SUÑER, J., 1928. Amer. J. Physiol., **83**, 554.

GAIRNS, F. W., 1930. Quart. J. micr. Sci., **74**, 151.

GARVEN, H. S. D., 1925. Brain, **48**, 380.

GOLGI, C., 1880. *Opera Omnia*, 1903, vol. i, p. 171.

GREGOR, A., 1904. Arch. f. Anat. u. Ent., p. 112.

GUTMANN, E., and YOUNG, J. Z., 1944. J. Anat., **78**, 15.

HINES, M., and TOWER, S. S., 1928. Bull. Johns Hop. Hosp., **42**, 264.

HINSEY, J. C., 1927. J. Comp. Neurol., **44**, 87.

—— 1934. Physiol. Rev., **14**, 514.

HOLMES, W., 1943. Anat. Rec., **86**, 157.

HUBER, C., 1900. Amer. J. Physiol., **3**, 339.

—— and DE WITT, L. M. A., 1898. J. Comp. Neurol., **7**, 169.

HURSH, J. B., 1939. Amer. J. Physiol., **128**, 131.
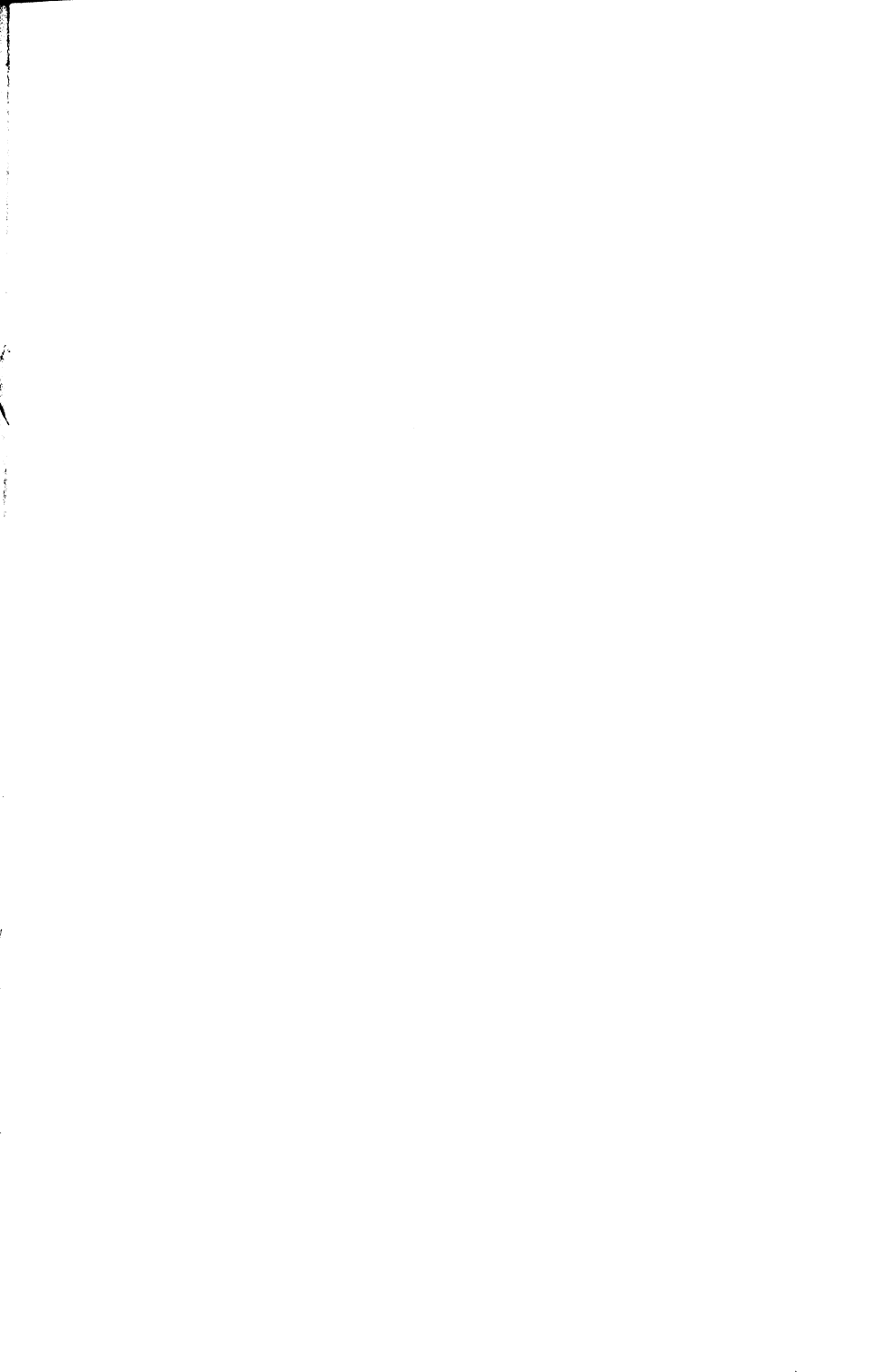
IRVINE, S. R., and LUDVIGH, E. J., 1936. Arch. Ophthal., **15**, 1037.
KATZ, B., and KUFFLER, S. W., 1941. J. Neurophysiol., **4**, 209.
KERSCHNER, L., 1888. Anat. Anz., **3**, 126.
—— 1893. Ibid., **8**, 449.
KÖLLIKER, A., 1889. *Handbuch der Gewebelehre des Menschen*, 6th ed. Vol. i. Leipzig.
KÜHNE, W., 1863. Virchows Arch., **28**, 528.
LANGWORTHY, O. R., 1924. J. Comp. Neurol., **36**, 273.
LEKSELL, L., 1945. Acta Physiol. Scandinav., **10**, Suppl. 31.
LIDDELL, E. G. T., and SHERRINGTON, C. S., 1924. Proc. Roy. Soc. B, **96**, 212.
LLOYD, D. P. C., 1943. J. Neurophysiol., **6**, 316.
MATTHEWS, B. H. C., 1931*a*. J. Physiol., **71**, 64.
—— 1931*b*. Ibid., **72**, 153.
—— 1933. Ibid., **78**, 1.
McCOUGH, G. P., and ADLER, F. H., 1932. Amer. J. Physiol., **100**, 78.
McINTYRE, A. K., 1941. Trans. Ophthal. Soc. Australia (Brit. Med. Ass.), **3**, 99.
MORSON, S., and PHILLIPS, G., 1937. J. Physiol., **88**, 199.
O'LEARY, J., HEINBECKER, P., and BISHOP, G. H., 1934. Amer. J. Physiol., **110**, 636.
PALLOT, G., 1934. Bull. d'hist. app., **11**, 337.
PERRONCITO, A., 1901. Arch. Ital. de Biol., **36**, 245.
—— 1902. Ibid., **38**, 393.
DE REZENDE, N., 1942. New York St. J. Med., **42**, 2124.
RUFFINI, A., 1893. Arch. Ital. de Biol., **18**, 106.
—— 1897. Brain, **20**, 368.
—— 1898. J. Physiol., **23**, 190.
SHERRINGTON, C. S., 1894. J. Physiol., **17**, 211.
SIHLER, C., 1895. Arch. mikr. Anat., **46**, 709.
SUTTON, A. C., 1915. Amer. J. Anat., **18**, 117.
TELLO, J. F., 1907. Trav. lab. rech. biol. univ. Madrid, **5**, 227.
—— 1917. Ibid., **15**, 101.
—— 1922. Zeit. ges. Anat., Abt. 1, Zeit. Anat. Ent., **64**, 348.
TOWER, S. S., 1932. Brain, **55**, 77.
VOSS, H., 1937. Zeit. f. mikr.-anat. Forsch., **42**, 509.
WEISS, G., and DUTIL, A., 1896*a*. C.R. Soc. Biol., **48**, 290.
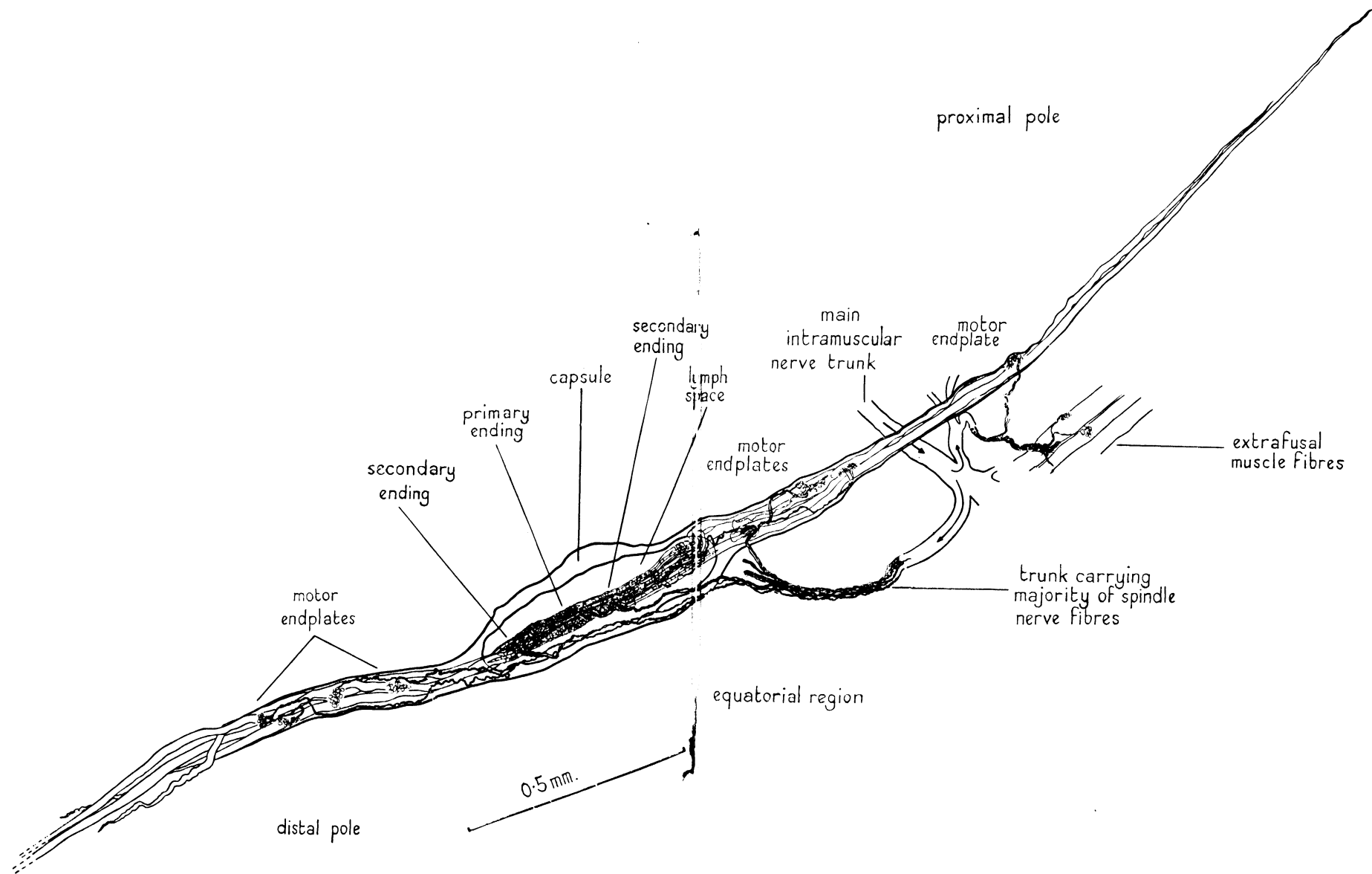—— 1896*b*. Arch. de Physiol., **8**, 368.

## DESCRIPTION OF PLATES

### PLATE I

A complex muscle-spindle of a rabbit from m. vastus intermedius reconstructed as described in the text. The figure has been reduced nine times from the original, which was over 8 feet long. Many features have been omitted (e.g. cross-striations, vascular supply, all nuclei other than those of motor end-plates and the central nuclei of the myotube regions and nuclear bags of the intrafusal muscle-fibres) in order to demonstrate the innervation to the best advantage. The extreme end of the proximal pole was attached to perimysial connective tissue; at the distal pole the two muscle-fibres whose ends could be traced were attached to the endomysium of extrafusal muscle-fibres. The spindle is fully described in the text and various portions of it are illustrated in more detail in Text-figs. 3–5, 8, 10–12; the object of this plate is merely to give a general view of the entire end-organ.

### PLATES II AND III: KEY TO LETTERING

*ax.sh.nuc* axial sheath nucleus; *b.v* blood-vessel; *br.pr.n.f* branches of primary nerve-fibre; *br.sec.n.f* branches of secondary nerve-fibre; *caps* capsule; *constr* intrafusal muscle-fibre constricted by nerve-ending; *extraf.m.e.p* extrafusal motor end-plate; *f.s* flower-spray type of ramification; *im.n.t* intramuscular nerve-trunk; *intraf.m.f(s)* intrafusal motor fibre(s); *l.sp* lymph space; *myt.rg* myotube region of intrafusal muscle-fibre; *myt.rg.nuc* central nuclei in myotube region of intrafusal muscle-fibre; *nuc.bag* nuclear bag; *p.pr.e* portions of primary ending; *plr.rg* polar region; *pr.e* primary ending; *pr.n.f* primary nerve-fibre; *sec.e* secondary ending; *sec.n.f* secondary nerve-fibre; *trab.conn.tiss* trabecular connective tissue.

proximal pole

secondary
ending

capsule

secondary
ending

lymph
space

main
intramuscular
nerve trunk

motor
endplate

primary
ending

motor
endplates

extrafusal
muscle fibres

secondary
ending

motor
endplates

trunk carrying
majority of spindle
nerve fibres

equatorial region

0·5 mm.

distal pole

D. BARKER—PLATE I

## PLATE II

Gold chloride preparations, Gairns's method.

Fig. 1. *Cat.* Complex muscle-spindle with one primary and two secondary sensory endings from m. vastus medialis.

Fig. 2. Primary ending of spindle in fig. 1. Part of the secondary ending which lies to the left of the primary is also shown.

Fig. 3. Secondary ending which lies to the right of the primary in the spindle shown in fig. 1.

Fig. 4. *Rabbit.* Intermediate type of spindle with one primary and one secondary sensory ending from m. vastus intermedius.

Fig. 5. The primary and secondary endings of the spindle in fig. 4.

## PLATE III

Silver preparations of spindles from m. vastus intermedius of rabbit; fig. 10 L.S. 20 $\mu$, Holmes's method, the remainder L.S. 25 $\mu$ de Castro's method.

Fig. 6. Nuclear bags of intrafusal muscle-fibres and portions of primary ending. In places the ramifications can be seen constricting the intrafusal muscle-fibres; they are closely applied to the 'sarcoplasmatic membrane' and lie underneath the sarcolemmal and axial sheaths.

Fig. 7. The spiral complex *s* of the secondary ending in Text-fig. 12 illustrated by optical sectioning; A lowest, E highest plane of focus. The two intrafusal muscle-fibres are kinked at one point.

Fig. 8. Part of a secondary ending showing two complete rings amongst the ramifications.

Fig. 9. Section showing the major portion of another secondary ending. Portions of the neighbouring primary ending lie to the left of the figure.

Fig. 10. Part of a primary ending; the turns of a loosely coiling spiral entwine one end of a nuclear bag and part of the myotube region of an intrafusal muscle-fibre, the typical location of this type of ramification. The nuclear bags lie to the right of the figure and the myotube regions to the left in the portions of the intrafusal muscle-fibres included in the section.
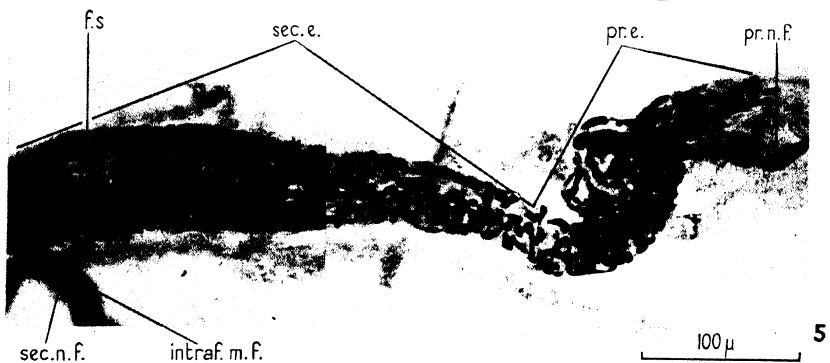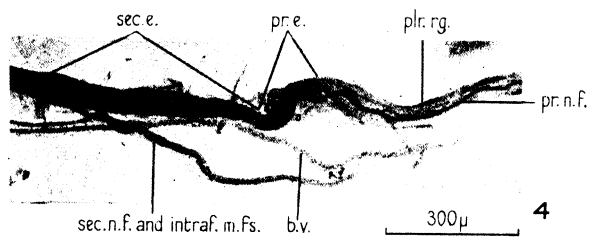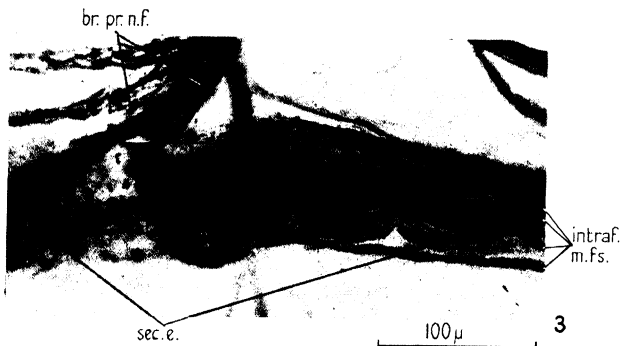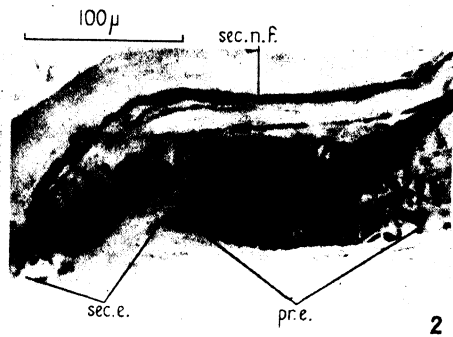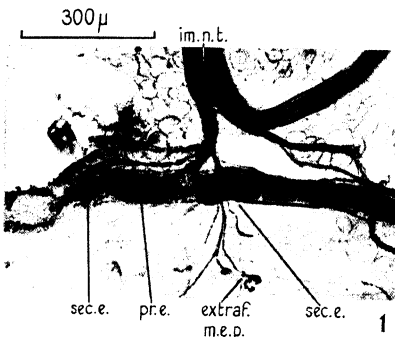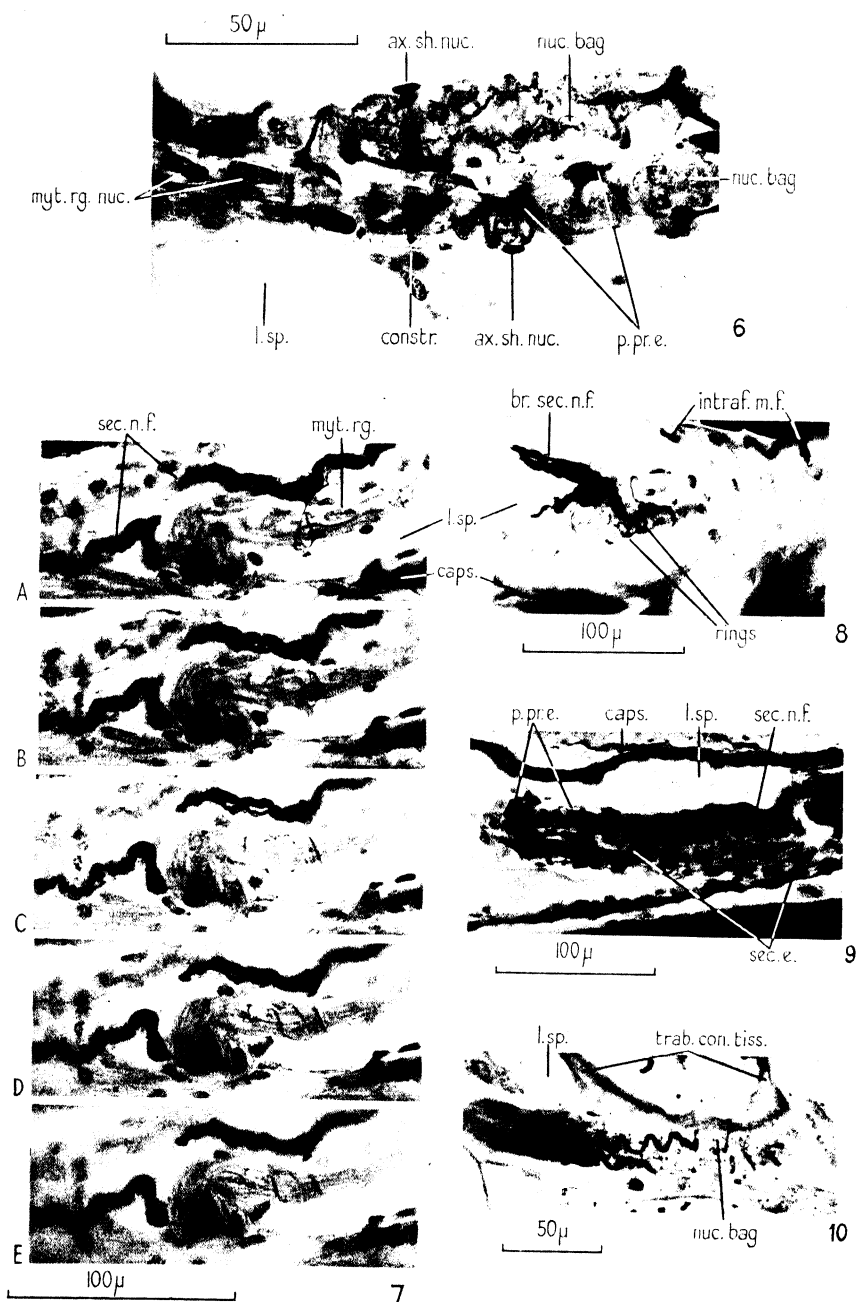
# The Cultivation of Adult Mammalian Skin Epithelium
## *in vitro*

BY

## P. B. MEDAWAR

*(From the Department of Zoology, University of Birmingham)*

**With five Text-figures**

THE purpose of this paper is to describe a simple technique for the short-term cultivation of adult mammalian skin epithelium *in vitro*. The conditions of cultivation are such as allow the rapid proliferation and migratory spread of skin epithelium while maintaining the characteristic functional activity and histological appearance of its cells.

The technique was devised to investigate the effect of iso-antibodies on cells. It requires little, if any, modification for use as an adjunct to routine biochemistry (e.g. in studying the influence of specific metabolic inhibitors on cell movement and division: cf. Medawar, 1947), or for submitting cells to the direct action of hormones. It has the disadvantage that the cultivated cells do not grow away from the explanted tissue into the culture medium, so that the direct microscopic study of living cells (an amenity to which, in conventional tissue culture, most others have been sacrificed) must be forgone. The tissue can, however, be studied in the transformed state in which it is most familiar to histologists: in stained sections.

### METHODS

One central principle of adult tissue culture was discovered by Parker (1936, 1937): many adult tissues require more oxygen than their embryonic precursors to initiate and maintain growth and functional activity. A second innovation of Parker's, likewise adopted here, was the culture of adult tissues for functional survival in fluid media. The long latent period that precedes the outgrowth of cells from adult tissue when it is embedded (or, as it may prove, embalmed) in a plasma jelly surrounded by air probably represents the time taken for adult cells to adjust themselves to growth at oxygen tensions lower than they have been accustomed to. Some adult tissues—spleen, for one—do not make this adjustment and usually die. On the other hand, it is shown here that adult skin epithelium will migrate vigorously and proliferate moderately at an oxygen tension no higher than that provided by a serous culture medium kept in gaseous equilibrium with air. At higher tensions, cell division quickens up and migratory activity is relatively subdued.

*Principle of the Method.* Thin, lightly vaselined, skin slices are floated raw side down in a stoppered test-tube or flask on 3–20 ml. serum kept in gaseous

equilibrium at 38° C. with air or an air-oxygen mixture by a smooth rotating or rocking motion. Cultures in high-oxygen media may be maintained up to 8 days, but preferably not beyond 6 days, without interference. By then the dermis may have become completely enclosed in epidermal epithelium that has migrated around it, the graft being sealed off by a layer of cuticle of its own formation. Cultivation beyond eight days would certainly require sub-culture by division and reopening of the encysted explant. Although there is no reason (except the increasing danger of infection) to doubt that it could be achieved, the prolonged cultivation of skin epithelium, being unnecessary for the purpose in hand as for many others, is not reported on here. Skin explants in a gas phase of air, in which migration is more vigorous, usually achieve complete self-encystment in 4 days, and the epithelium farthest from the free surface tends to show ischaemic changes thereafter.

Skin and culture media from adult rabbits have been used throughout. The culture fluid need not be taken from the skin donor. Explanted skin will indeed grow with undiminished vigour in the presence of body fluids, tissues, and tissue extracts from rabbits heavily and specifically immunized against it (Medawar, 1948).

*Preparation of Skin for Culturing.* Strict cleanliness can be achieved but asepsis can only be aspired to: the method of skin preparation described in full by Medawar (1947) has been adhered to throughout. The most convenient size for explants is a $3 \times 4$ mm. rectangle or a $3 \times 3$ mm. square. The thinner they are the better; but, although routine work has been confined to the use of explants only 0·5 mm. thickness, minute 'pinch grafts' as thick as $1\frac{1}{2}$ mm. centrally and tapering down to epidermal thickness at the edge have been used with success.

Between removal and explantation it is convenient to lay the skin fragments raw side down in a Petri dish containing a filter paper damped with Ringer's solution.

*The Culture Medium.* Serum has been used as the basis of the culture medium in the majority of tests; either plain, or as an extractive for minced adult tissues. Krebs-Ringer-bicarbonate containing adult tissue extract, or defibrinated whole blood diluted with four times its volume of clear serum, have both been found to support 4 days' vigorous growth in a gas phase of air.

Serum is most conveniently prepared by spinning down spontaneously clotted whole blood which has been received directly into a centrifuge tube from the median ear artery in dilatation (Medawar, 1946). Alternatively, the blood can be defibrinated immediately upon removal by twirling in it a fine glass rod with a ∩-shaped ending.

Whatever the chosen medium, eight volumes have as a routine measure been diluted with 1 vol. 5 per cent. glucose in $H_2O$ and 1 vol. streptomycin solution, 200 u.ml. in Ringer. Streptomycin and glucose solutions were steri-lized by passage through a no. 5 porosity sintered glass filter and stored in sealed 1-ml. ampoules at room temperature awaiting use. Before using

streptomycin as an internal antiseptic, on the recommendation of Dr. E. S. Duthie, approximately 50 per cent. of all cultures of 4 days' standing were lost by infection. No 4-day culture has been lost since its adoption, and only some 20 per cent. of cultures prolonged to 8 days in a single vessel. Strictly controlled tests have failed to reveal that streptomycin has any harmful effect on cells at a final concentration of 20 u./ml. in the culture medium, and concentrations as high as 100 u./ml. have been used.

It goes without saying that all equipment has been sterilized and all tissues have been handled with the precautions necessary for culture work.

Since the skin fragments are floated on the surface of a culture medium kept constantly in motion, the volume of the medium and its depth are less important than they would be with the use of submerged tissues in stationary media. By using large volumes of culture fluid, the special precautions taken by Parker (1936, 1937) to maintain a 'physiological' pH were found to be unnecessary. (Parker cultivated up to 100 mg. spleen tissue in as little as 2 ml. fluid, and kept the pH of the medium within reasonable bounds by using an excess of $NaHCO_3$ in the culture fluid in combination with a controlled excess of $CO_2$ in a periodically refreshed gas phase.) In our present experiments 11 independent glass-electrode determinations of the final pH of 4-day rocker flask cultures in a high $O_2$ gas phase gave a mean of pH $7 \cdot 37 \pm 0 \cdot 08$ standard error. Six independent determinations of the final pH of similar cultures of 8 days' standing gave a mean of pH $7 \cdot 21 \pm 0 \cdot 10$.

*Cultivation in a Gas Phase of Air.* This technique has been found suitable for skin, for epithelium from the kidney cortex, and for the duct elements (not the 'parenchyma') of liver and submaxillary gland. It is not suitable for lymph node or spleen. The behaviour *in vitro* of tissues other than skin will be described by Medawar (1948).

In all experiments using a gas phase of air (hereafter 'air cultures' for short), skin slices have been floated two or three at a time on 3 ml. culture fluid in 16 × 130 mm. Pyrex glass test-tubes with B-19 size standard ground-glass stoppers sealed down with stopcock grease and held firmly in place by elastic bands (Text-fig. 1a). In the apparatus illustrated by Text-fig. 1b, up to eight such tubes may be rotated simultaneously at a tilt of 15° from the horizontal in a water-bath at 38° C. Save that it is adapted for use in a water-bath, as short-period culture work requires, the apparatus illustrated differs only in details of design from the standard 'roller-tube' instrument introduced into tissue culture by Gey (1933).

The tubes should be given a sharp tap if the skin slices show a tendency to become beached on the side wall of the test-tube during the early stages of rotation. Sometimes the fragment is beached during part of its circuit and is picked up and floated during the remainder. This has proved to be unobjectionable.

Two or more skin fragments may often coalesce with each other marginally during cultivation, without appreciably impairing the growth of either one. For some purposes (cf. Medawar, 1948) it may be desirable to study the effect of one tissue on another by glueing the two together before cultivation. The

following procedure has been uniformly successful. Withdraw blood from the median ear artery and at once mix 4 ml. with 0·4 ml. 4 per cent. Na citrate. Spin down the corpuscles and decant the plasma. Mix a few drops of the plasma with one-tenth of its volume (or, if need be, more) of a 1 per cent. aqueous solution of anhydrous $CaCl_2$. Soak both tissue-pieces for a few
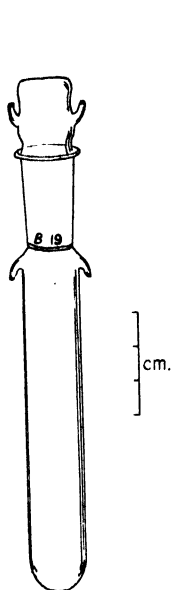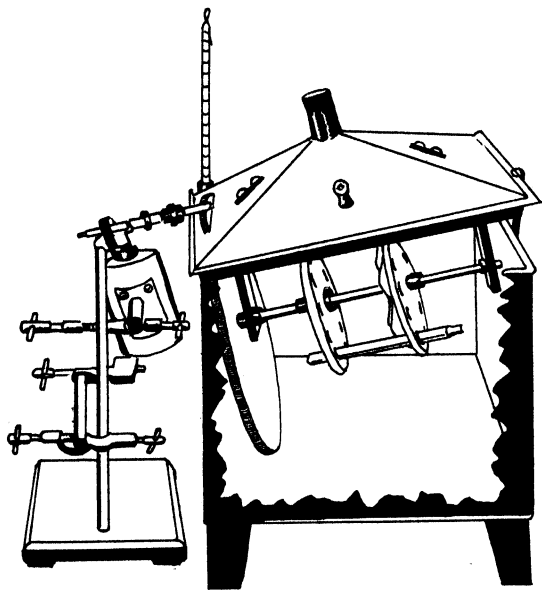


FIG. 1a  FIG. 1b

TEXT-FIG. 1. Apparatus for 'roller-tube' culture: (a) the culture tube, and (b) the rotating apparatus. The rotating unit is attached to the lid of a standard bacteriological water-bath: during running it is fixed by a spring strut at a tilt of 15° from the horizontal. A stout rubber washer attached to one of the two perforated plates prevents the tubes slipping in the tilted position. It will be noted that the long axis of each culture tube remains parallel to and equidistant from the central shaft during rotation. The drive is external, and connects by a rubber bush to the large milled driving-wheel which protrudes through the lid. A resistance in series with the motor is used to maintain rotation at about 6 times per minute.

seconds in the mixture, lay them together in the disposition required, and allow about a minute for clotting to take place.

*Cultivation in a High-oxygen Gas Phase.* Cultures of this type are most conveniently done in conical flasks fitted with accessories for gas perfusion. Two sizes of flask have been used: of 50-ml. capacity, for fluid volumes up to 4 ml., and of 250-ml. capacity (Text-fig. 2a) for volumes up to 20 ml. The larger flask differs from the smaller only by having a stoppered side-tube to facilitate the introduction and removal of culture media and tissues. Being designed for periods of culture in relation to which 'warming-up time' would be negligible, the flasks were mounted in a dry-air incubator, upon a platform smoothly rocking 15° from the horizontal either way about four times per minute (Text-fig. 2b).
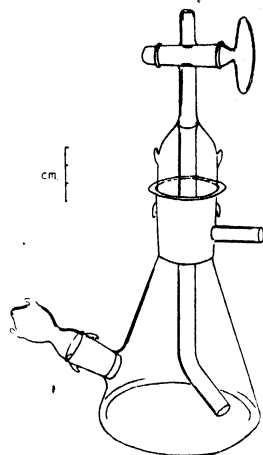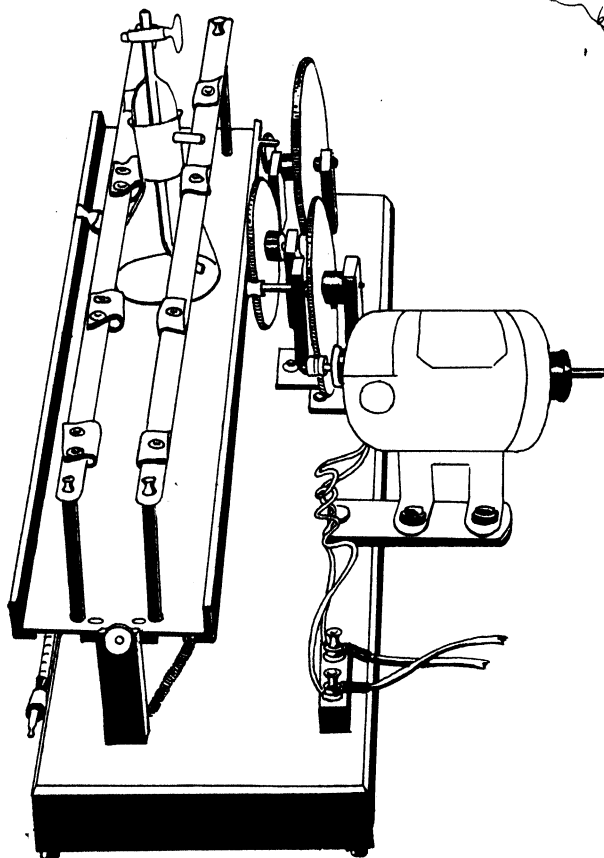
FIG. 2a



FIG. 2b

TEXT-FIG. 2. Apparatus for 'rocker-flask' culture: (a) the 250-ml. culture flask, and (b) the rocking device. The gas exit-tube on the neck of the culture flask is opened and closed by rotating the flask head. A small motor, suitably geared down to a cam, rocks the spring-loaded platform 15° from the horizontal either way about its long axis.

Oxygen being denser than air, the gas entry tube was prolonged towards the bottom of the flask. The exit tube, closed by rotation of the flask head, was connected to an ordinary water-displacement gas volume meter. In the writer's own experiments the exact oxygen tension of the cultivation fluid was of no importance, and sufficient uniformity was achieved by passing through the flask one measured volume of cylinder $O_2$ at the timed rate of 100 ml. per minute, giving a final concentration of 65–70 per cent. It is clear that if oxygen-nitrogen-$CO_2$ mixtures of known composition are to be introduced, at least five volumes should be passed through the flask to flush out residual air.
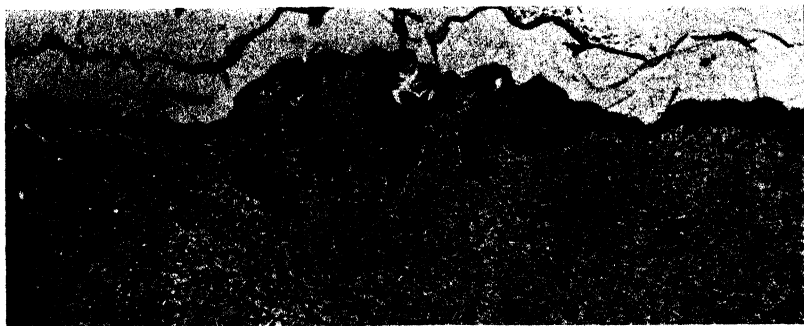


Text-fig. 3. Illustrating the outcome of a 'survival test'. The skin culture, after 4 days *in vitro*, was transplanted back to a raw area on the chest of its donor, and removed 10 days later. The original explant is represented by the raised-up central portion of the section: its continued survival is proved by the widespread outgrowth of strongly hyperplastic epithelium from it. Ehrlich's haematoxylin and eosin; × 27.

*Survival Tests; Histological Methods.* When skin has been cultured in a medium suspected to affect its growth adversely, it has been found informative and reassuring to duplicate each skin slice, one being reserved at the end of the experiment for histological examination, and the other subjected to a routine test of its continued survival. Any histological method appropriate to skin can be used on skin cultures, but to avoid messiness the serum still sticking to the explants on their removal should be rinsed away with Ringer's solution before fixation.

The principle of the routine survival test is to graft the cultured skin back to a raw area on the animal from which it originally came. Outgrowth in that position constitutes an absolute proof of continued survival; necrosis indicates with slightly less complete certainty that the fragment was in fact dead. The technique has been described in full by Medawar (1947). Since a surviving explant may well have encysted itself completely, it is essential that the delicate epithelial jacket over the dermal surface should be scraped away before grafting. The unmistakable outcome of a positive survival test 10 days after grafting is illustrated by Text-fig. 3.

## RESULTS

Cell migration is faster in air than in high-$O_2$ cultures, and complete self-encystment (Text-fig. 4 *a*, *b*) occurs correspondingly sooner and more often. By 24 hours the shoulders of the culture are rounded and smoothed off by the



FIG. 4*a*
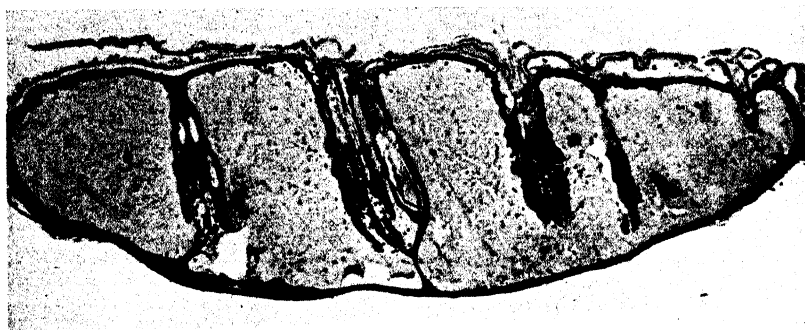


FIG. 4*b*

TEXT-FIG. 4. Illustrating the complete self-encystment by migratory overgrowth of skin squares cultivated for 4 days in roller-tubes (gas phase: air) in serum (*b*) and serum with tissue extract (*a*). The survival of dermal mesenchyme cells is apparent in (*a*). In both the process of self-encystment has been helped by the breaking through of follicle epithelium on to the dermal surface. (The culture media were in both cases derived from a rabbit specifically immunized against the skin explanted from the donor: cf. Medawar, 1948.) Ehrlich's haematoxylin and eosin; × 47.

beginnings of migratory overgrowth, and complete self-encystment, at first always by a delicate monolayer of epithelial cells, may be achieved as early as the third day. The 'wave front' of the epithelial sheet thus advances at a rate of the order of 1 mm. per day. Migratory overgrowth may be surreptitiously helped by follicle epithelium breaking through on the dermal side of the skin slice. Though at first only a monolayer (Text-fig. 5), migratory epithelium

becomes in due course stratified, and the process of stratification occurs earliest in the regions over which migration occurred first. The epithelium is squamous, but, as with hyperplastic skin, the cuticle stays recognizably cellular and is not reduced to mere flakes.

Growth in plain serum has been found to be uniformly inferior to growth in serum which has been used as an extractive for adult spleen, lymph nodes, kidney tissue, or leucocytes.



TEXT-FIG. 5. Illustrating the strong epidermal proliferation and relatively subdued migratory activity of a skin square rocked for 4 days in 10 ml. serum containing lymph-node fragments in a 'high-$O_2$' gas phase. A cell monolayer of epithelium has crept from the left of the two follicles and is beginning to grow over the dermal surface. Survival of dermal mesenchyme cells is evident. (As in the cultures illustrated by Text-fig. 4, the serum and lymph-node fragments were taken from a rabbit specifically immunized against the skin explanted from the donor.) Ehrlich's haematoxylin and eosin; × 88.

Epiboly is not the only evidence of cell migration, for, as with skin grafts *in vivo* (Medawar, 1944), there is an upward migration of follicle epithelium to the skin surface which opens out the follicle necks and eventually results in the throwing off of the hairs carried over with the explant. In fact, with 'overgrowth' deputizing for 'outgrowth', a skin explant *in vitro* behaves just like a skin graft transplanted to a raw area large enough to permit outward spread of epithelium from the graft centre. Correspondingly, the histological appearance of explanted skin epithelium is hardly distinguishable from that of ordinary hyperplastic skin.

Cell division in air cultures occurs in all regions of the epithelium, including the newly formed, except at its extreme lip. An extensive series of mitotic counts (Medawar, 1948) has shown that it is slightly more frequent at the

thickened shoulders of the culture than elsewhere. The mean frequency to be expected in an air culture when fixed after 4 days' residence *in vitro* is of the neighbourhood of one mitotic figure per mm. of superficial epidermis per 8 $\mu$ section.

Epiboly, and migratory activity in general, is less vigorous in high $O_2$-cultures than in those run in air. Cell division, on the other hand, is more rapid, so that the superficial epithelium comes to acquire the deep stratification characteristic of strongly hyperplastic skin (Text-fig. 5). The continued formation of normal cuticle and the persistence of sebaceous gland epithelium (Text-fig. 5) is evidence that the characteristic functional activity of skin epithelium has not been impaired.

Besides epithelial elements, fibroblasts, monocytes, and vascular endothelium survive and proliferate in cultured skin, whether in a high-oxygen gas phase or in air. The collagen fibres retain their normal packing better in air cultures than in high-oxygen cultures, possibly because in the former they are more rapidly protected by the overgrowth of an epithelial sheath. In high-oxygen cultures of 6 or 8 days' standing the fibres sometimes swell and become slightly waterlogged in appearance (Text-fig. 5); and this is associated with a tendency for the epidermis to part from the dermis in odd patches where the dermal papillae are 'oedematous'.

## DISCUSSION

The fine opportunities that the technique of tissue culture offers for the study of living cells under the microscope has caused it to develop along somewhat narrow lines. The study of cultured cells under the microscope requires not merely growth but *out*growth. Outgrowth is easier to achieve by cultivation in blood-plasma jellies than otherwise. Embryonic tissues, with their relatively low demands on oxygen, grow far better in plasma jellies than their adult successors. The association of tissue culture with the growth of embryonic tissues in plasma, or at least with growth in plasma, has therefore come to be regarded as little short of obligatory. Alternatively, it has come to be widely believed that growth 'does not occur' in fluid media, or that the growth of adult tissues, being slow of inception and dilatory in progress, renders them unsuitable for systematic use.

The experiments described here combine with those of Parker (1936, 1937) to show that the rapid organized growth of adult tissues in fluid media is so easy to achieve as to put the method within reach of any laboratory dealing with problems for which tissue culture technique might offer hope of solution. Since virtually unlimited quantities of adult tissue can be cultivated with no more difficulty than is entailed by the provision of flasks large enough to hold them, it is to be hoped that the metabolism of growing and moving cells will come to be studied by more direct methods than hitherto.

Plastic Surgery, Oxford University. The photographs were taken by Mr. D. A. Kempson, and the drawings made by Miss Jean Morpeth.

## SUMMARY

Adult skin epithelium migrates and proliferates strongly when incubated at body temperature in a stirred serous fluid medium in gaseous equilibrium with air or an air-oxygen mixture.

## REFERENCES

GEY, G. O., 1933. Amer. J. Can., **17,** 752.
MEDAWAR, P. B., 1944. J. Anat. Lond., **78,** 176.
—— 1946. Brit. J. exp. Path., **27,** 15.
—— 1947. Quart. J. micr. Sci., **88,** 27.
——— 1948. Unpublished observations.
PARKER, R. C., 1936. Science, **83,** 579.
—— 1937. Ibid., **85,** 292.

# The Structure and Deposition of the Cuticle in the Adult Mealworm, *Tenebrio molitor* L. (Coleoptera)

BY

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**With two Plates and eight Text-figures**

IN three earlier papers (Wigglesworth, 1933, 1945, 1947*a*) the structure and deposition of the cuticle of *Rhodnius prolixus* have been described. The main conclusions reached were as follows. The epicuticle is a composite structure made up of four layers: (i) a 'cuticulin' layer composed of a condensed lipoprotein subsequently tanned by quinones; (ii) a layer of material rich in dihydroxyphenols; (iii) a thin layer of crystalline, orientated wax molecules responsible for the waterproofing of the cuticle (cf. Beament, 1945); and (iv) an outermost 'cement' layer protecting the wax.

The lipoproteins which form the foundation of the epicuticle are apparently synthesized by the oenocytes before being transferred to the epidermal cells. The oenocytes reach their maximum development immediately before the cuticulin layer is deposited and then diminish rapidly in size. The polyphenol layer is secreted from the epidermal cells by way of the pore canals which appear to penetrate the cuticulin layer. The wax is secreted in the same way immediately before moulting. The cement layer is poured out from the dermal glands over the surface of the wax immediately after moulting.

The hardening which takes place shortly after moulting is due to the tanning of the lipoprotein of the epicuticle and the cuticular protein of the exocuticle by quinones produced by the oxidation of the dihydroxyphenols (Pryor, 1940*b*; Pryor, Russell and Todd, 1947). The endocuticle is laid down by the epidermal cells during the next few days.

In the deposition of the cuticle the epidermal cells first separate from the old cuticle and undergo active mitosis. Many more cells are produced than are required. Consequently, great numbers of them suffer autolysis with the formation of the 'chromatic droplets', until, finally, a regular epithelium with the nuclei evenly spaced is produced, which then proceeds to lay down the new cuticle (Wigglesworth, 1943*a*).

If the cuticle of *Rhodnius* (and many other insects) is gently rubbed with alumina dust, the cement layer and the wax layer are abraded; the layer containing the polyphenols is then exposed and will stain a deep chestnut-brown if the insect is immersed in ammoniacal silver hydroxide. The polyphenol layer is likewise exposed more or less readily by extraction of the cuticle surface with chloroform and other wax solvents.

The ease with which the polyphenols are exposed by these two procedures varies greatly in different insects and in different parts of the same insect. In adult beetles it was found that there is no detectable abrasion (or, at least, no exposure of silver-reducing materials) over the hard regions of the cuticle in insects left in contact with alumina dust (Wigglesworth, 1947*b*). Silver staining after this treatment is confined to the soft dorsal cuticle of the abdomen and to the various intersegmental membranes.

The object of the present work was to compare the structure and formation of the cuticle in these different regions of the body in the adult beetle *Tene-*
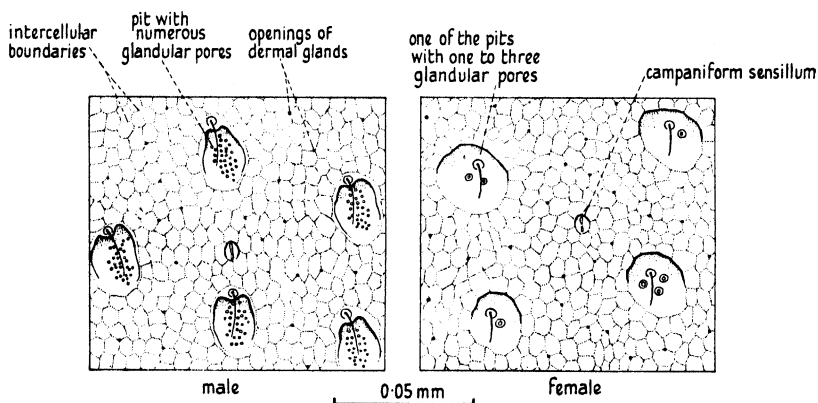


TEXT-FIG. 1. Surface view of ventral abdominal cuticle of *Tenebrio* adult.

*brio*, and to see how far the conclusions arrived at from the study of the *Rhodnius* cuticle were applicable to this unrelated insect.

## † STRUCTURE OF THE ADULT CUTICLE AND EPIDERMIS

The dorsal cuticle of the abdomen, where it is covered by the elytra, is excessively thin (total thickness 4 $\mu$). The tergites are distinguishable only by their shining surface and faintly amber tint which contrasts with the colourless matt surface of the lateral and intersegmental regions. The varied sculpturing and spicules of the different parts will not be described.

The ventral cuticle is very hard and a deep amber-brown in colour (total thickness in an old insect 36 $\mu$). Evenly dispersed over the sternites are shallow oval depressions each with a tiny slender bristle curving backwards from its anterior end (Text-fig. 1). In the floor of these pits are minute pores—1–3 in the female, a closely packed group of about 25–30 forming a diminutive 'pore plate' in the male. In both dorsal and ventral cuticle the limits of the epidermal cells are clearly indicated in the sculpturing of the cuticular surface. There is a shallow groove between each cell area.

Both tergites and sternites are pierced by the ducts of dermal moulting glands; in the sternites these occur as a circle of some 8–10 glands around

each pit, but they are seen with difficulty in ordinary preparations. They occur also in the pleural regions.

Text-fig. 2 shows histological sections of the dorsal and ventral integument as seen in a recently moulted adult about 6 days old. The cuticle of the tergites (Text-fig. 2A) consists of an excessively thin 'epicuticle', an unstained exocuticle about 1·5 $\mu$ thick, and an endocuticle of about 2·5 $\mu$, but no further details can be seen. In the sternites (Text-fig. 2B) the cuticle is made up of a fairly thick amber-coloured 'epicuticle', an amber exocuticle about 12 $\mu$ in
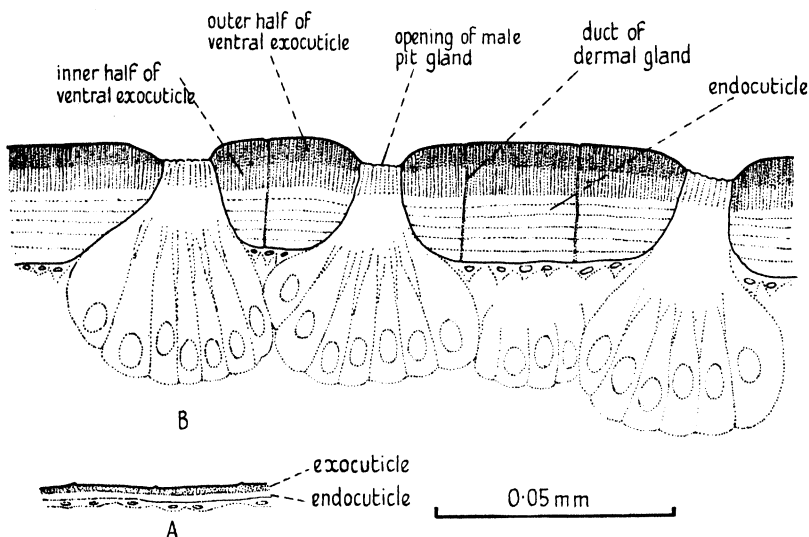


TEXT-FIG. 2. Sections through the cuticle of young adult male, about 6 days old. A, dorsal cuticle; B, ventral cuticle.

thickness in which the vertical striation of the pore canals is just visible, and a laminated acidophil endocuticle which, in an insect several weeks old, may be double the thickness of the exocuticle. The exocuticle is differentiated faintly into an outer and inner half. The outer half has a slightly greyer shade in the unstained state, and in the recently moulted adult it stains bluish with haematoxylin. At intervals the cuticle is pierced by the ducts of dermal glands, and in the male the groups of pores in the pits form conspicuous interruptions in the sections.

There is the usual complex of cellular elements in the epidermis, best seen in the recently moulted adult (Text-fig. 3). On the sternites, besides the epidermal cells, there are scattered oenocytes, the dermal moulting glands (unicellular glands with some three other cells forming the duct, &c. (Hundertmark, 1935)), and the pit glands. Each pore of the pit glands is connected with a single large cell. In the mature male these cells become enormously swollen and the glands project like closely packed buttons far beyond the

surface of the shrunken epidermis.[1] In the female, with usually not more than two pores, the associated gland cells are comparatively inconspicuous.

The structure of the cuticle, particularly the ventral cuticle, has been studied by the methods used on *Rhodnius*.

(i) *Fresh Sections.* In sections cut with the freezing microtome and examined fresh in water the pore canals appear much as in stained sections.
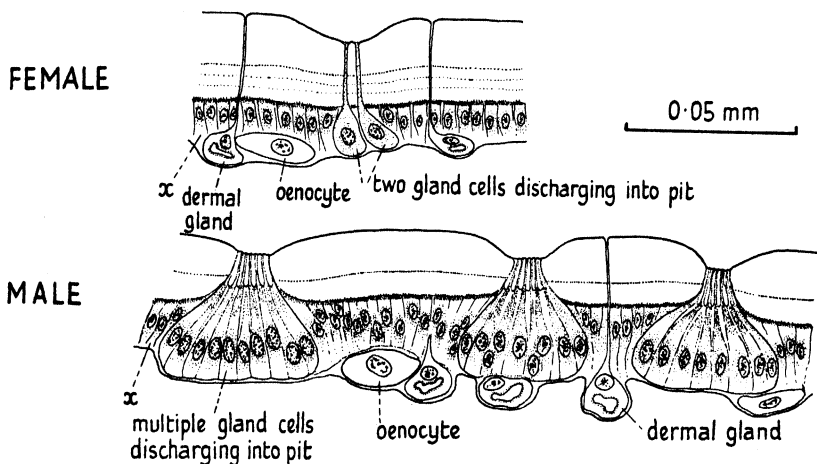


TEXT-FIG. 3. Sections through ventral integument of adult *Tenebrio* 2 days after moulting. *x*, epidermal cells with basiphil apical staining and basiphil filaments extending into the pore canals.

(ii) *Fresh Sections treated with Ammoniacal Silver.* Similar sections, 10 $\mu$ thick, were immersed for 1 hour in 5 per cent. ammoniacal silver hydroxide and mounted in Canada balsam. All the pore canals in the exocuticle run a parallel vertical course. In the outer half of the exocuticle their contents stain brown, but only where they come very close to the exposed surface of the sections or where the canals have been actually cut open (Text-fig. 4). These filaments are almost certainly in the form of a close spiral (as described by Richards and Anderson, 1942, in the cockroach), but only here and there can this be resolved with certainty with the light microscope. In the endocuticle the pore canals run a somewhat uneven spiral course, often changing direction as they cross from one lamina to the next and converging gradually towards the base. Throughout the endocuticle they contain a black deposit

[1] These glands resemble those described by Hoffbauer (1892) in the elytra of the Cerambycid *Tetropium*. Hoffbauer noted great differences in closely related species but makes no mention of sexual dimorphism. The glands in *Tenebrio* must presumably have some sexual function. Perhaps in the male they produce an aphrodisiac secretion. It is pointed out to me by Dr. H. E. Hinton of the British Museum, in a personal communication, that hair tufts, presumably the outlets of glands, occur in many male Coleoptera. In other parts of the body (legs, prothorax, &c.) the male *Tenebrio* has only two or three pores in the pits, like the female.

of silver along their course; and this is not limited to the canals exposed on the surface of the sections.

The staining of the pore canal contents is seen even more clearly if the sections after treatment with the silver are bleached for 24 hours in perhydrol; and then it is seen that the exposed surface of the exocuticular matrix also stains weakly with the silver. Beyond the limit of the brown-staining pore canals in the exocuticle there is an 'epicuticle' apparently devoid of pore canals; but in many places the brown filaments come so close to the surface that it is not possible to ascribe a measurable thickness to this layer.
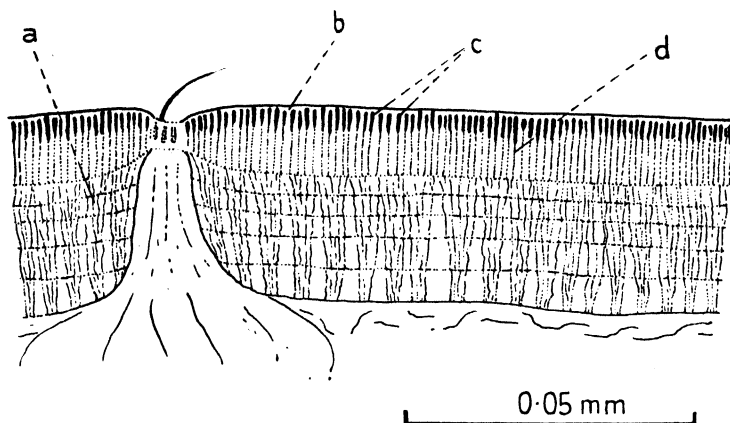


TEXT-FIG. 4. Section through ventral cuticle of mature adult *Tenebrio*, cut with freezing microtome, stained with ammoniacal silver hydroxide and bleached with hydrogen peroxide. *a*, converging convoluted pore-canals in the laminated endocuticle; *b*, 'epicuticle', for the most part apparently free from pore canals; *c*, pore canals in the outer half of the exocuticle with deep brown silver-staining contents; *d*, pore canals in the inner half of the exocuticle without silver-staining contents.

(iii) *Fresh Sections dried in Air.* Fresh sections were dried thoroughly in warm air and then mounted direct in Canada balsam. The pore canals of the endocuticle contain air in many places and appear as black threads. In the exocuticle they contain no air.

(iv) *Silver Staining after Abrasion with Alumina.* If the intact insect is immersed in ammoniacal silver hydroxide no staining of the cuticle occurs. If it is rubbed gently on filter paper dusted with alumina and then immersed in the silver solution, the dorsal cuticle shows a uniform pinkish-brown staining over all the raised points, with the tips of the pore canals, extending apparently into the substance of the brown-staining membrane, seen in surface view as minute black points. The ventral cuticle shows no staining with silver after abrasion with alumina except at the intersegmental membranes which stain in the same manner as the dorsal cuticle.

(v) *Silver Staining after Scratching with Glass.* If the hard parts of the ventral cuticle are treated more brutally by scratching, with varying degrees

of severity, with a fragment of glass and then immersed in ammoniacal silver, it is again found that where the injury is very superficial the scratches are scarcely visible and there is no silver reduction. Where the scratches go deeper the distal ends of the pore canals stain dark brown in a pale brown background. (There are occasional areas where there is a uniform pale brown staining and the pore canals show up as white points. The explanation of this, which does not appear to be an optical effect, is uncertain.)
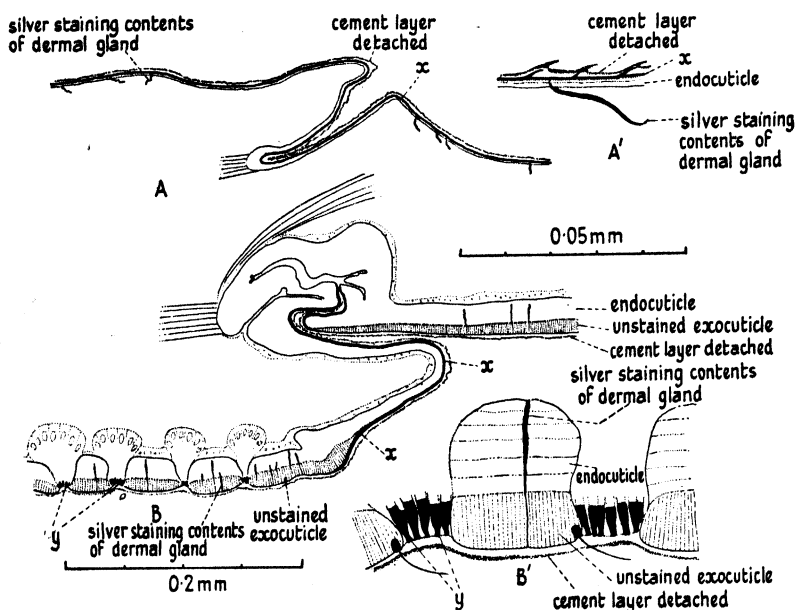


TEXT-FIG. 5. Longitudinal sections of cuticle of adult male *Tenebrio*, 1 week after moulting, boiled 5 minutes in chloroform and then treated with ammoniacal silver hydroxide. A, dorsal cuticle and intersegmental joint; A', detail of same; B, ventral cuticle. and intersegmental joint; B', detail of same. *x*, silver-stained epicuticle and outer exocuticle; *y*, silver-stained cuticle lining the openings of the male pit glands.

(vi) *Silver Staining after Chloroform Extraction.* Extraction with chloroform likewise leads to the exposure of the silver-staining material much more readily in the same regions which are affected by abrasion with alumina. This is most clearly demonstrated by boiling the insect for 5 minutes in chloroform before immersion in the ammoniacal silver. Text-fig. 5 shows longitudinal sections of the dorsal and ventral abdominal cuticle in a *Tenebrio* adult so treated.

The dorsal epicuticle, including the spicules, stains everywhere an intense brown. The same staining extends into the outer part of the exocuticle—sometimes as a uniform brown, sometimes limited to pore canals. The inner half of the exocuticle (stained red with Mann's methyl blue eosin) and the blue-staining endocuticle show no silver reduction.

In the ventral integument, on the other hand, silver staining of the epicuticle is limited to the outer folds of the intersegmental membranes. Elsewhere (apart from the 'cement layer' which will be discussed later) there is no silver staining of epicuticle or exocuticle except in the lining of the orifices of the pit glands or 'pore plates'. The residual contents of the dermal moulting glands also stain deeply and are very conspicuous after this treatment.

(vii) *Chitosan Test.* The ventral cuticle of the fully hardened adult some weeks old was treated with saturated potassium hydroxide at 150° C. until just colourless, then washed in alcohol and examined under a coverslip in surface view. In the deeper layers (endocuticle) the criss-cross fibrils of chitin running in different directions in successive layers are clearly seen; and the areas corresponding with each epidermal cell are evident. In the superficial layers (exocuticle) the pore canals are readily visible; they appear evenly distributed and the cell limits cannot be seen. Now that the amber material has been dissolved away, the pore canals, as seen in optical section, are open to the surface.

If acid iodine is allowed to diffuse slowly in below the coverslip, the matrix of the cuticle in the superficial layers takes on a violet colour, but as this colour spreads it is quite evident that the pore canals remain colourless as minute white points. There is certainly no chitinization of the contents of the pore canals of the exocuticle.

(viii) *Disintegration in Nitric Acid and Potassium Chlorate.* The abdominal cuticle was boiled in chloroform for 10 minutes and then immersed in concentrated nitric acid saturated with potassium chlorate. The dorsal cuticle shows little ebullition as the thin endocuticle dissolves. A very thin epicuticle is left which on warming breaks up with the liberation of small oily droplets.

In the ventral cuticle there is much evolution of gas as the inner layers are dissolved. On warming fairly strongly the exocuticle then fuses and disperses in the form of feebly refractile spheres. If at this stage the preparation is washed with 50 per cent. alcohol and flooded with Sudan B in 70 per cent. alcohol, very few of the droplets stain. They consist presumably of broken-down protein and chitin. There are, however, a few fat-staining droplets, particularly where the epicuticle is beginning to disintegrate. If the heating of the preparations is continued the epicuticle remains last; and as it breaks up, undoubted oil droplets, highly refractile and staining with Sudan B, appear in great quantity. They are far more copious than in the dorsal cuticle, a fact which agrees with the much greater thickness of the epicuticle on the ventral surface as seen in sections.

(ix) *Demonstration of Wax and Cement Layers.* It has not been possible to demonstrate a wax layer in the cuticle of the mature *Tenebrio* adult; the evidence for the presence of this layer will be given when the deposition of the cuticle is described. The existence of a cement layer is readily demonstrated.

If the legs or elytra are immersed fresh in xylene, droplets of water slowly exude from the epicuticle (Wigglesworth, 1942). In many places during this

process a thin layer flakes away in fragments; in other places a delicate continuous membrane, the 'cement layer' is lifted from the surface.

This layer can also be demonstrated by silver staining. If the mature adult beetle is boiled in chloroform for 5 minutes and then treated with ammoniacal silver, a delicate membrane can be seen over the surface of the sternites, extending into the bristle-bearing pits. It consists of very fine evenly dispersed brown granules in a colourless substance. Where the cuticle has been rubbed in mounting it is torn and partially removed; and in sections of such insects it is detached and very easily seen.

Around the openings of the dermal glands, in some preparations, there is a non-staining disk devoid of granules (Pl. I, fig. 1). And extending from the orifice of a certain number of glands (always the glands at the centre of the spaces between the pits, which are a little larger than the others) there is a brown-staining convoluted filament lying on the surface of the cuticle (Pl. I, fig. 2). This appears to be the extruded residue of the secretion from the glands.

A series of adult beetles, 2 weeks after moulting, were subjected to extraction with boiling chloroform for periods of 1, 5, 15, 30, and 60 minutes before treatment with the silver. Insects untreated with chloroform showed no silver staining. All the others showed the granular cement layer over the sternites. The staining was often most intense in the furrows between the cell boundaries on the cuticle surface, giving a net-like appearance (cf. Pl. II, fig. 10). In the insects extracted for longer periods disintegration of the membrane was more advanced; the granules had fused in many places to give larger silver-staining spheres and the convoluted filaments from the dermal glands showed the same change (Pl. I, fig. 3).

It thus appears that the cement layer, as was suggested in *Rhodnius*, also consists of tanned material; but that the silver-reducing groups of the polyphenol concerned are not accessible until the material has been subjected to boiling chloroform. Presumably it is compounded in some way with lipides.

(x) *Summary of the Conclusions on the Structure of the Cuticle.* From these varied observations we may conclude that the dorsal cuticle is not only excessively thin compared with the ventral cuticle, but the substance of the outer layers (the epicuticle and the very thin exocuticle of the delicate sclerites), like the same parts in *Rhodnius*, still contain polyphenol material in a state accessible to the silver solution if the cuticle is cut or the protective coverings on the surface are removed by abrasion or by extraction with lipid solvents.

The thick and brittle cuticle of the sternites has very different properties. It has a relatively thick and hard epicuticle which will not reduce ammoniacal silver (when immersed for 1 hour) even when exposed by abrasion, section, or lipid extraction. The exocuticle is likewise hard and impermeable; polyphenol material easily accessible to the silver solution is confined to the contents of the outer parts of the pore canals, and the limitation of silver staining to the surface of the fresh sections shows how impermeable is the

exocuticle to the diffusion of silver through its substance. (The black deposits in the pore canals of the endocuticle may well be simply a precipitate of the silver by salt or protein.)

The state of the material in the lumen of the pore canals is difficult to determine. In the endocuticle many of the canals contain air on drying; but as Dennell (1947*b*) points out, this does not disprove the existence of solid filaments within them. In the exocuticle they appear to be solid; perhaps their contents consist of protein which has been tanned along with the substance of the exocuticle during the hardening process. It is certain that they do not contain filaments of chitin.

As will be shown later a wax layer covers the cuticulin of the epicuticle, and over the surface of this is a cement layer which likewise consists of tanned materials in intimate association with lipides. In ordinary sections neither cement nor wax layer are optically distinguishable from the cuticulin layer. It has not, therefore, proved possible to homologize the structures here described with the inner and outer epicuticle as defined by Dennell (1946) in *Sarcophaga* larvae and in *Tenebrio* and other adult beetles.

DEPOSITION OF THE NEW CUTICLE IN THE PUPA AND YOUNG ADULT

*Outward Changes in the Pupa and Young Adult*

Newly moulted pupae of *Tenebrio* were kept at 25° C. Formation of the adult beetle then takes place according to the following time-table. During the first 24 hours the pupa has a glassy appearance and the eyes show only brown central points in the facets; no separation of the adult is visible in the living pupa. By the second day the adult is just beginning to separate from the pupal cuticle, for example, at the tips of the appendages. At 3 days the separation is obvious in the claws, palps, &c., but the new cuticle has not begun to appear. The eyes are just beginning to darken. By 5 days the eyes are fairly dark and the new cuticle is well defined. By 6 days the eyes are quite dark; the claws, tarsal segments, the femero-tibial articulations, and the distal third of the mandibles are darkening. By 7 days the head and legs are becoming dark generally. Moulting to the adult occurs on the eighth day.

The last stages of moulting can be roughly timed by observing the sheaths of the appendages. By the end of the seventh day the limbs are chestnut-brown in colour but the pupal sheaths enclosing them are fully distended with moulting fluid. About 6–10 hours before moulting to the adult, as the inner layers of the pupal cuticle are digested and the moulting fluid absorbed, the sheaths of the last pair of legs begin to collapse, followed by the middle and anterior pairs. Finally, the sheaths around the palps, labrum, and mandibles crumple and collapse, and moulting usually takes place from 2 to 4 hours later. By this time the moulting fluid has disappeared, the pupal cuticle is excessively thin and fragile, and the surface of the insect is quite dry.

The newly emerged adult has the head and prothorax amber; the legs, particularly the joints and claws, are likewise amber, and so are the margins and posterior extremity of the abdomen; but the tergites and sternites of the

abdomen, and the elytra, are alike colourless. Within a few hours the beetle becomes amber all over. After 1 day the general colour is pale chestnut; after 2 days, dark brown; and after 3 days it is fully darkened and almost black.

### Histological Changes in the Epidermis and Cuticle

The histological changes have been observed in serial transverse sections of the abdomen fixed with Carnoy's and Bouin's fixatives at all stages, stained with haematoxylin, Mann's methyl blue and eosin, and Mallory's triple stain. Likewise at all stages the dorsal and ventral integument of the abdomen have been removed, freed from underlying tissues, and mounted flat after (i) fixation in Carnoy and staining with haematoxylin; (ii) fixation in Bouin and staining with Sudan black B; (iii) fixation in Altmann and counterstaining with carmine.

There is no interruption in the process of growth when *Tenebrio* pupates. Within less than 24 hours after moulting mitosis in the epidermis of the sternites has already begun. At this stage there is no obvious difference between the cuticle and epidermis of the dorsal and ventral walls of the abdomen (Text-fig. 6A, *a* and *b*), apart from the fact that oenocytes are confined to the sternites. The epidermis is thin and the nuclei lie all in one plane, not nearly contiguous. The oenocytes of the ventral wall still occur in pairs, embedded among the epidermal cells (Text-fig. 6A, *c*).

By *one day* after pupation a difference is apparent between the epidermis of the dorsal and ventral integument. In the sternites the epidermal cells are now much more numerous, the nuclei are so crowded that they lie in several planes, and there are abundant mitoses. Certain of the nuclei are in chromatolysis (Pl. I, fig. 5). The whole epidermis is in such a violent state of flux that the centres of formation of dermal glands, bristles, &c., cannot be recognized. The oenocytes are still mostly in pairs; they are enlarging slightly and now lie wholly on the inner surface of the epidermis (Pl. I, fig. 6). In the dorsal integument there are no mitoses, no oenocytes, and, as yet, little chromatolysis.

By *two days* (Text-fig. 6B) the same differences between the dorsal and ventral epidermis are becoming exaggerated. That on the dorsal integument is still extremely thin and many of the nuclei are breaking down so that chromatic droplets in all stages of formation are abundant (Pl. I, fig. 4).

By *three days* (Text-fig. 6C) mitosis and chromatolysis in the ventral epidermis is still in active progress; the dermal glands are becoming visibly differentiated and the oenocytes are becoming large and acidophil. In the dorsal epidermis chromatolysis is well advanced and, since no mitosis has occurred, the cells are becoming very sparse as well as very attenuated.

By *four days* (Text-fig. 6D) the epidermis has been detached from the old cuticle, chromatic droplets have almost disappeared, the nuclei are evenly distributed, and the oenocytes are large, lobulated, and strongly acidophil (Pl. I, fig. 7). The innermost layer of the old cuticle stains with haematoxylin
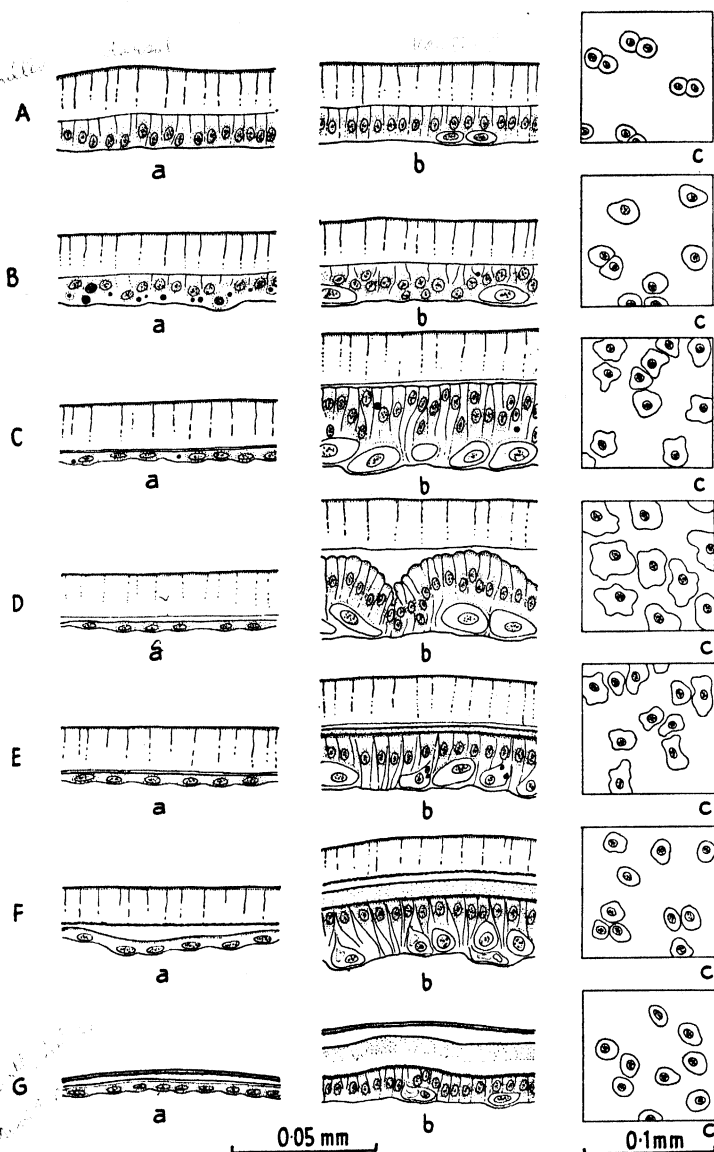
TEXT-FIG. 6. The formation of the adult cuticle in the pupa of *Tenebrio*. *a*, section of dorsal integument; *b*, section of ventral integument; *c*, surface view of oenocytes below the ventral epidermis. A, newly moulted pupa; B, pupa at 2 days; C, 3 days; D, 4 days; E, 5 days; F, 6 days; G, shortly before moulting to adult. The details are given in the text.

and the secretion of the new cuticle is imminent. Particularly in the ventral epidermis the cells now show distinct intercellular membranes and in many places these have a remarkably exact hexagonal arrangement (Pl. I, fig. 8). In preparations fixed with Altmann the great lobulated oenocytes stain dark grey; and they stain conspicuously in Sudan black B.

By *five days* (Text-fig. 6E) (occasionally by 4 days) the new epicuticle is formed. It is refractile and eosinophil, excessively thin on the dorsal surface as compared with the ventral. In ordinary histological sections it appears to be homogeneous although the outer extremity of the cell body lying immediately below is seen to be vertically striated. In the Altmann preparations, in which it can be studied in surface view or in optical section, the epicuticle stains grey and the pore canals are clearly visible as colourless threads in a dark matrix; their outer ends are closed only by an excessively thin refractile membrane. The oenocytes are still large but do not stain so deeply in Altmann.

By *six days* (Text-fig. 6F) the formation of the exocuticle is in progress and the oenocytes are becoming reduced in size; many of them are clearly dying with chromatolysis of their nuclei. The new cuticle, particularly the epicuticle, still stains grey with osmic acid. The arrangement of the epidermal cells, as seen in surface view, is not so regular, but they still lie in rows, this being associated no doubt with the deposition of the parallel strands ('Balken') of chitin extending from cell to cell. Highly vacuolated dermal glands occur in both dorsal and ventral integument.

By *seven days* the exocuticle of the ventral integument is becoming much thicker and its inner and outer halves can be distinguished. For example, with Mallory's stain the inner half stains blue and the outer half stains red, being traversed by blue-staining pore-canal fibrillae which can be traced almost if not quite to the surface of the epicuticle. The dermal moulting glands are even more distended and vacuolated and the pit glands in the male are beginning to enlarge. The oenocytes are reduced in size and stain only a faint grey in osmium tetroxide. The osmic staining of the epicuticle has almost disappeared. Digestion of the inner layers of the pupal cuticle is beginning and is completed by the eighth day (Text-fig. 6G).

In the *young adult* there are no very striking histological changes. The regular arrangement of the epidermal cells is largely lost, but in many places the cells, as seen in surface view, still lie in parallel rows with filaments extending from one to the next as the criss-crossing chitin strands of the endocuticle are formed. As the endocuticle is being secreted the outer parts of the cells stain deeply with haematoxylin, and blue-staining filaments extend into the inner ends of the pore canals. The endocuticle is just about complete in 4 days after moulting. By that time the epidermal cells are small and shrunken and the pit glands of the male are greatly swollen and project far below the epidermis (see Text-fig. 2).

In addition to the sub-epidermal oenocytes described above there are clusters of large oenocytes segmentally arranged in the neighbourhood of the

spiracles (Koch, 1940). These appear to go through the same cycle as the oenocytes below the epidermis. They reach their maximum size at about the fourth day of pupal development and appear then to be discharging their secretion into the blood.

### Formation of the Epicuticular Layers

(i) *Polyphenol Layer*. The formation of the cuticulin layer of the epicuticle takes place as we have seen between the fourth and fifth days after pupation by the secretion of lipoprotein from the epidermal cells. Upon this is deposited a layer of material rich in silver-reducing substances, the 'polyphenol' layer.

If the epidermis with its new epicuticle, 5–6 days after pupation, is immersed directly in the ammoniacal silver solution there is a clear-cut silver precipitate marking out the membrane between the cell bodies. At the outer limit of each cell there is some finely punctate silver staining, but the epicuticle does not stain. Here and there are single cells in which the outer region stains intensely with the silver (Pl. I, fig. 9) and from the strongly staining cells black filaments run through the pore canals of the newly formed epicuticle to give discrete and evenly distributed black points on the surface.

Soon the black points become general, though always confined to the limits of the cells. It is often possible when examining these preparations in surface view to note the grouping of the pore canals in optical section, to follow them upwards, and to observe that the black droplets on the surface of the cuticle have the same grouping. There can be no doubt that the droplets are formed, as was claimed in *Rhodnius*, by the extrusion of material from the pore canals (Pl. I, figs. 10, 11).

Gradually the exuded droplets enlarge and fuse, so that in pupae in which the new cuticle has been exposed and treated with the silver during the seventh day all intermediate stages can be seen leading up to a condition where the epicuticular cap of each cell is covered by a deep-chestnut-staining layer, more or less continuous, with the punctate black staining of the tips of the pore canals below (Pl. I, fig. 12). Finally this layer joins up with that covering adjacent cells until the cell boundaries almost disappear. At no stage does this superficial polyphenol layer extend over the floor of the pits. Consequently, these show up as oval white spots in the preparations (Pl. I, fig. 12; Pl. II, figs. 4, 5, &c.).

If, during this process, the exposed cuticle, before treatment with the silver, is wiped with a piece of filter-paper cut to a point, the silver-staining material will run together into rounded droplets. If it is more strongly rubbed with filter-paper, the material may be removed completely, leaving only the black-staining tips of the pore canals.

These observations, made upon whole preparations seen in surface view, can be confirmed in sections. Text-fig. 7 shows sections of the new cuticle which had been exposed in the 6-day pupa and treated with silver. In Text-fig. 7A the cuticulin layer is visible as a clear zone beyond the silver-staining

filaments in the exocuticle. But here and there these filaments cross the clear zone, becoming continuous apparently with the polyphenol droplets on the surface. At the stage represented in Text-fig. 7B the polyphenol is being actively secreted; it appears chiefly in the form of tapering filaments passing outwards through the cuticle, with their pointed end leading, to fuse upon the surface. The form of these filaments bears a striking resemblance to that assumed by droplets of biliverdin as they pass through the striated border of the Malpighian tubes in *Rhodnius* (Wigglesworth, 1943*b*).

(ii) *Wax Layer*. During the last few hours before moulting the polyphenol layer is largely covered over. Soon after the silver staining has reached its
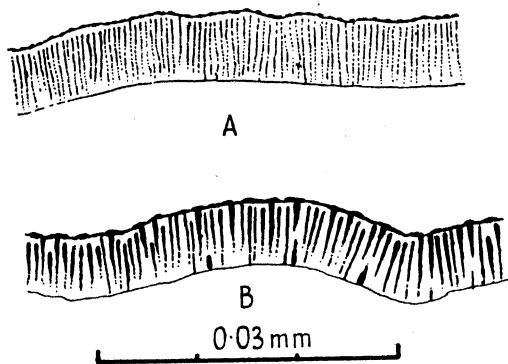


TEXT-FIG. 7. Section of new cuticle in 6-day-old pupa. Whole insect immersed for 1 hour in ammoniacal silver hydroxide before fixation. Description in text.

greatest intensity and become practically continuous, it begins to break up into rounded patches with non-staining areas between; and gradually the staining spots become reduced in number and in size (Pl. II, fig. 1). Around each spot can be seen a pale-brown halo. This is due to the diffuse staining of the exocuticle and the dark-brown staining of the filaments in the pore canals, the distal limits of which now lie on a distinctly lower plane than the dark-brown spots (Pl. II, fig. 2)—though sections through the dark spots often show brown filaments running from them into the pore canals.

These observations show that at this stage silver can get into the deeper layers of the cuticle only through the silver-staining patches; the intervening areas are impermeable. Having got in it will diffuse laterally through the exocuticle to reach adjacent pore canals. The slightest abrasion of the surface with alumina, or brief immersion in chloroform, will expose the polyphenol layer everywhere.

It would appear that, as in *Rhodnius*, the polyphenol layer is being covered by a layer of lipoid or wax immediately before moulting. At the time of moulting droplets of water will not leave a waxed pipette to adhere anywhere to the surface of the adult cuticle; but after extraction for 5 minutes in boiling chloroform wetting is notably increased. At this stage there is still plenty of

silver staining in the form of scattered rounded spots, particularly along the side of the abdomen and on the intersegmental membranes. Here and there the stained spots lie in rows, mostly running in an antero-posterior direction (Pl. II, fig. 3). These have evidently resulted from abrasion during moulting and show how fragile is the protective layer at this time. Within 2 hours the silver staining is much reduced, though what remains has the same distribution as before. By 6 hours after moulting there is a further marked diminution, but even after 24 hours there are occasional scattered spots on the intersegmental and marginal regions.

(iii) *Cement Layer.* Meanwhile the cement layer is being poured out over the surface of the wax. At the time of moulting, droplets of water, at the tip of a very fine waxed pipette, will not adhere to the surface of the sternites. After 1 hour they usually begin to adhere slightly, particularly in the lateral regions. When brought into contact with the cuticle the droplet on the pipette breaks and a very tiny drop remains on the insect. After 6 hours the droplets will adhere all over the cuticle with an angle of contact ranging from 90° upwards, but they never spread over the surface. This slightly increased affinity for water takes place in spite of the fact that the hardening process that is going on in the substance of the cuticle will be tending to make it hydrophobe; it is certainly the result of the outpouring of the cement layer.

The cement comes from the dermal glands. We have seen that after extraction with boiling chloroform it will stain with ammoniacal silver, and this property may be utilized to demonstrate its secretion. Adult *Tenebrio* in the act of moulting and at periods of 30 minutes, 1 hour, 2 hours, &c., afterwards have been fixed briefly in Carnoy, boiled in chloroform for 5 minutes, and then immersed in the ammoniacal silver.

At the time of moulting the sternites stain deeply after this treatment; excepting the floor of the pits, the dark-brown colour is continuous and extends for the most part right across the abdomen. The contents of the dermal glands stain black (Pl. II, figs. 4, 5, and 6). The gland vesicle has a convoluted form tapering to a point, and in many of the glands the secretion appears to be pressed into a distended mass near the orifice. Towards the sides of the abdomen in some places the dense polyphenol staining of the exocuticle is becoming discontinuous (Pl. II, fig. 7); and where that happens the dermal glands are partially emptied and there is some superficial dark-brown silver staining, concentrated particularly in the furrows between the cell areas. Occasionally, around the mouths of the ducts of individual glands, there are rounded areas where the staining of the exocuticle is becoming covered; clearly the glands are just beginning to extrude their secretion.

At 30 minutes after moulting there is hardly any increased wetting of the cuticle by water. Staining with ammoniacal silver now shows, particularly in the lateral regions, that the cement is being discharged, with the result that the polyphenol layer is no longer exposed by chloroform extraction. Where this process is most advanced the glands are empty; where the polyphenol staining is still continuous, as in the middle of the segments, the glands are

distended as at moulting; and all intermediate stages occur (Pl. II, figs. 8 and 9). The new cement layer shows regular fine brown granules in a colourless matrix.

One hour after moulting only the middle regions of the sternites show intense silver staining. The lateral parts stain a diffuse brown. In the central area the dermal glands still show intense black contents. In the lateral part they are completely empty, the polyphenol layer is entirely covered, and the only silver staining is in the granular cement. This extends over the whole surface, including the floor of the pits and the bristles, but it is most intense in the furrows between the cellular impressions. Here it may form more or less continuous brown lines, giving a net-like appearance (Pl. II, fig. 10). In the intermediate zone occasional dermal glands can be seen in which the black-staining contents are in process of discharge.

After 6–8 hours the dermal glands are mostly emptied and the cement layer is evident everywhere. There is little change at 1 day after moulting. At 2 days after moulting the black-staining filaments discharged from the largest of the dermal glands have made their appearance (Pl. I, fig. 2) and practically all the glands are emptied.

It was interesting to apply this technique of staining with silver after extraction with boiling chloroform to *Rhodnius* at the time of moulting. In the *Rhodnius* fifth-stage nymph there are two sorts of dermal glands (Wigglesworth, 1933): type 'B' present in great numbers, particularly around the bristle-bearing plaques, with a large distended oval vesicle; and type 'A' much less plentiful, with an elongated intracellular vesicle. In the nymph at the time of moulting it is only type 'A' whose contents stain black with silver after chloroform extraction; the vesicle contents of type 'B' are unstained. If the preparation is allowed to dry on the slide after dehydrating in alcohol the vesicles of type 'B' fill with air and on mounting in Canada balsam they show up very conspicuously.

In order to see whether glands of this type had been overlooked in *Tenebrio* a preparation of a newly moulted *Tenebrio* adult was likewise allowed to dry in the air, but no glands were revealed. It appears that in *Tenebrio* there is one type only, and its contents reduce silver after chloroform extraction.

It appears from these observations that the cement layer in *Rhodnius* resembles the substance of the oötheca in the cockroach, as described by Pryor (1940a), in being the product of two glands, one of which secretes a protein solution and the other a polyphenol. When the cement in *Rhodnius* is first discharged it is strongly hydrophil and droplets of water spread actively on the surface (Wigglesworth, 1947a). In *Tenebrio* there is only a slight increase in this adhesion of water when the cement is secreted. This is probably because the cement in *Tenebrio* contains more lipoid material; for it is the product of a single type of gland, the contents of which reduce silver only after extraction with boiling chloroform.[1]

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[1] It is unlikely that the glands opening into the floor of the pits in *Tenebrio* contribute to the cement. There is a very great difference in their form and development in the two sexes and they do not appear to reach full activity until some days after moulting.

## Hardening of the Cuticle

No attempt has been made to follow the movements and distribution of the enzymes concerned in the hardening and darkening of the cuticle (cf. Dennell, 1947*a*).

The most striking feature of the hard ventral abdominal cuticle of *Tenebrio* as compared with the cuticle of *Rhodnius* is the failure of the epicuticle and of the substance of the exocuticle to reduce silver once hardening is complete.[1]

Text-fig. 8 shows a fresh section of the cuticle in the young adult one day after moulting, when hardening is still far from complete, cut with the freezing
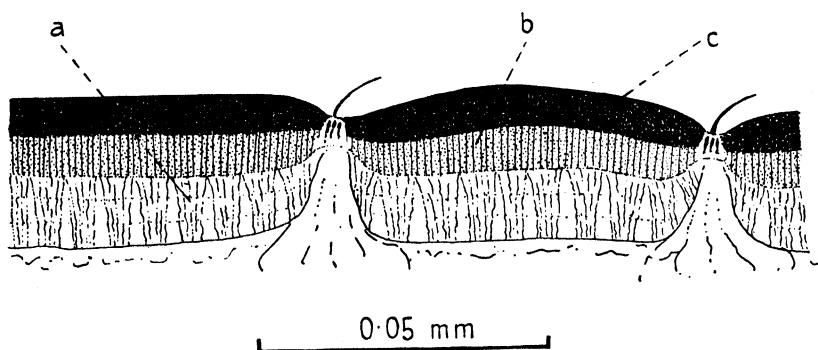


TEXT-FIG. 8. Section of ventral abdominal cuticle of adult *Tenebrio* 1 day after moulting, cut with the freezing microtome and immersed fresh in ammoniacal silver hydroxide. *a*, laminated endocuticle partly formed; pore canals slightly converging; *b*, inner half of exocuticle showing rather feeble silver staining; *c*, outer half of exocuticle showing intense silver staining.

microtome and treated with ammoniacal silver. The outer half of the exocuticle is stained a uniform intense blackish-brown in which the pore canals can scarcely be differentiated. The inner half also stains brown, but not so deeply, and the pore canals are more distinct. The endocuticle is about equal in thickness to the exocuticle. The lamination is faintly visible and the slightly converging pore canals are brown-stained throughout their course. The much stronger silver reduction in the outer half of the exocuticle agrees with the limitation of polyphenol to the outer half of the pore canals in the fully hardened cuticle (p. 200). Perhaps this outer half represents the amount of exocuticle that has been laid down at the stage when the most active secretion of polyphenol is taking place.

The process of hardening in the sternites is most readily studied by rubbing the cuticle surface gently with alumina before immersion for 1 hour in the silver (Wigglesworth, 1945). If this is done at the moment of moulting the wax layer is removed and everywhere there is a strong diffuse staining of

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[1] This difference is one of degree. Immersion in ammoniacal silver for 1 hour has been used for the test, and this gives only a very faint staining of sections of the exocuticle. On prolonged immersion more intense reduction would doubtless be obtained.

the exocuticular matrix and an intense black staining of the pore canals (Pl. II, fig. 11). At 6 hours after moulting, when the cuticle is just beginning to darken, the abraded areas stain dark brown as before, but in the form of small spots instead of extensive rounded patches (Pl. II, fig. 12). The exocuticle still stains readily, but now the cement has been laid down the covering is less fragile. At 1 day after moulting the silver staining after abrasion consists of faint pink areas with the punctate brown staining of the tips of the pore canals distributed through them, while in the fully hardened beetle at 4 days, as we have seen, there is no silver staining of the sternites after abrasion with alumina.

These results indicate that although silver-reducing material is probably still present in the epicuticle and exocuticle, the substance of these layers in the fully hardened insect has become so impermeable that the silver does not have access to it.

Along with the hardening, the exposed parts are blackened. If fragments of the fresh cuticle of the newly moulted insect are immersed in dilute ferric chloride, the exocuticle develops a diffuse violet coloration. This is most evident in the parts which will become darkened. Perhaps it indicates the concentration of tyrosine in the cuticle of these regions. The cuticle removed at an earlier stage (in pupae at 6 or 7 days) and immersed in dilute ferric chloride gives a diffuse greenish coloration which changes to red in the presence of alkalies. This colour is said to be given by ortho-dihydroxy-phenols only when they are in solution (Lison, 1936). No definite coloration has been obtained with the 'polyphenol layer' on the surface of the cuticulin. Perhaps the phenol responsible for the silver reduction is bound to protein and is insoluble.

### Waterproofing of the Cuticle at the Time of Moulting

Pupae of *Tenebrio* were kept at 25° C. in dry air and weighed daily. Among 12 pupae with an average weight of 97·7 mg. (85–119) the loss of weight per diem was about 1·3 mg. The loss in the young beetles after moulting ranged from 1·2 to 1·8 mg. per diem with a mean of about 1·6 mg. During the 24 hours which included the moult the loss of weight (excluding the dry weight of the cast skin) varied widely from 4·1 to 12·6 mg., with an average of 7·7 mg.

In three of these insects weighings were made during the act of moulting in order to estimate the relative importance of the water lost with the skin and that lost by transpiration afterwards. The results were as follows:

|  | A | B | C |
|---|---|---|---|
| Total loss of water during 24 hours from moulting, in mg. | 11·8 | 6·4 | 8·0 |
| Water lost with the skin | 4·2 | 3·0 | 2·5 |
| „    „    by transpiration | 7·5 | 3·4 | 5·5 |
| Weight of the dry cast skin | 1·2 | 1·5 | 1·1 |

Weighings of insects at intervals after moulting showed that the increased rate of transpiration occurs chiefly in the early hours after shedding the skin; but here again there is great individual variation. For example, the insect B

above, which lost 3·4 mg. by transpiration in 24 hours, lost 1·2 mg. in the first 6 hours, 2·2 mg. in the next 18 hours; while the insect C, which lost 5·5 mg. in 24 hours, lost 3·2 mg. in the first 3 hours and 2·3 mg. in the next 21 hours.

These results agree with the observations on the newly moulted *Tenebrio* which show a variable amount of silver staining that disappears during the 24 hours after moulting. They are entirely consonant with the view that waterproofing results from the deposition of a layer of wax over the polyphenol.

In *Rhodnius*, during the day on which moulting occurs, the water loss is rather more than doubled (Wigglesworth and Gillett, 1936). Smallman (1942), in the case of *Dixippus*, found that the rate of loss was increased about four times but returned to normal within one day. The values in *Tenebrio* likewise show an increased loss on the day of moulting about four to six times that of the beetle during the succeeding days.

SUMMARY

The conclusions on the structure of the cuticle in *Tenebrio* have been summarized on p. 204.

Observations on the deposition of the cuticle are in general agreement with those made on *Rhodnius*.

Mitosis and chromatolysis precede the formation of the definitive epidermis. The basic layer of the epicuticle, 'cuticulin', is then laid down. It consists of condensed lipoproteins (subsequently tanned, it is supposed, by quinones) and its deposition is immediately preceded by the peak in the secretory cycle of the subepidermal oenocytes.

Pore canals from the epidermal cells penetrate the cuticulin layer and pour out silver-reducing material (believed to be dihydroxyphenols in insoluble form) upon its surface. This material is confined to the areas overlying the cell bodies during all but the last stages in its formation, when it fuses to give a more or less continuous layer.

During the last few hours before moulting a wax layer appears to be laid down over this polyphenol layer. By the time moulting occurs the polyphenol layer is almost covered and the insect is nearly waterproof. During the first day after moulting, while the secretion of the wax is being completed, the loss of water by transpiration is about four to six times the normal.

Very soon after moulting the dermal glands discharge the cement layer over the surface of the wax. The substance of this layer and the contents of the dermal glands reduce ammoniacal silver after extraction with boiling chloroform. It is suggested that it consists of polyphenol-containing material associated with protein and lipides.

(It is shown that in *Rhodnius* the cement layer is formed by the admixture of secretion from the two types of dermal gland previously described. The

one produces a solution of protein, the secretion of the other agrees in properties with that here described in *Tenebrio*. The similarity of this arrangement to that discovered by Pryor in the colleterial glands of the cockroach is pointed out.)

In addition to these cement glands there are glands of unknown function opening into the floor of the pits in the cuticle. These are highly developed in the sternites of the male, small and inconspicuous in the female.

## REFERENCES

BEAMENT, J. W. L., 1945. J. exp. Biol., **21**, 115.
DENNELL, R., 1946. Proc. Roy. Soc. B, **133**, 348.
—— 1947a. Ibid., **134**, 79.
—— 1947b. Ibid., 485.
HOFFBAUER, C., 1892. Z. wiss. Zool., **54**, 579.
HUNDERTMARK, A., 1935. Z. Morph. Oekol. Tiere, **30**, 506.
KOCH, A., 1940. Ibid., **37**, 38.
LISON, L., 1936. *Histochimie Animale*. Paris (Gauthier-Villars).
PRYOR, M. G. M., 1940a. Proc. Roy. Soc. B, **128**, 378.
—— 1940b. Ibid., 393.
——, RUSSELL, P. B., and TODD, A. R., 1947. Nature, **159**, 399.
RICHARDS, A. G., and ANDERSON, T. F., 1942. J. Morph., **71**, 135.
SMALLMAN, B. N., 1942. Proc. Roy. Soc. Edin. B, **61**, 167.
WIGGLESWORTH, V. B., 1933. Quart. J. micr. Sci., **76**, 270.
—— 1942. Bull. Ent. Res., **33**, 205.
—— 1943a. Quart. J. micr. Sci., **83**, 141.
—— 1943b. Proc. Roy. Soc. B, **131**, 313.
—— 1945. J. exp. Biol., **21**, 97.
—— 1947a. Proc. Roy. Soc. B, **134**, 163.
—— 1947b. Proc. Roy. Ent. Soc. Lond. A, **22**, 65.
—— and GILLETT, J. D., 1936. Ibid., **11**, 104.

## EXPLANATION OF PLATES

### PLATE I

All figures at the same magnification with 4-mm. objective except figs. 9, 10, and 11 taken with 2-mm. objective.

Fig. 1. Surface view of ventral abdominal cuticle. Boiling chloroform 5 minutes, followed by ammoniacal silver hydroxide. The black spots are the ducts of the male pit glands (cf. Text-fig. 5y). The cement layer is visible as a granular film; it shows colourless round areas and at the centre of each of these is a black point which is the opening of a dermal gland.

Fig. 2. The same. Towards the left the silver-stained cement layer has been partially rubbed off, leaving unstained areas. Silver-staining convoluted filaments protrude from the mouths of some of the dermal glands; others appear as small black spots.

Fig. 3. The same, but treated in boiling chloroform for 30 minutes. The cement layer is fusing into rounded silver-staining droplets. Above and below are convoluted filaments which have partially fused.

Fig. 4. Epidermis of dorsal abdominal integument in the 2-day-old pupa seen in surface view. Ehrlich's haematoxylin. Many of the nuclei are breaking down with the formation of chromatic droplets.

Fig. 5. Epidermis of ventral abdominal integument in the 1-day-old pupa seen in surface view. Ehrlich's haematoxylin. Numerous mitoses; abundant and crowded epidermal cells; some nuclei breaking down to form chromatic droplets.

Fig. 6. The same. More superficial view (from inside) showing the oenocytes, many of them still in pairs. Numerous chromatic droplets.

Fig. 7. The same in 4-day-old pupa showing the oenocytes very large and lobulated

Fig. 8. The same, showing the very regular arrangement of the nuclei of the epidermis. These cells are partially obscured by the large oenocytes which overlie them.

Fig. 9. The epidermis and new epicuticle of the abdominal sternites in the 5-day-old pupa treated with ammoniacal silver hydroxide. In the lower part the finely granular silver staining of the cells can be seen. The polygonal black areas are individual cells staining intensely with the silver. Black filaments run upwards from them into the new cuticle.

Fig. 10. Surface view of the new ventral cuticle in 6-day-old pupa treated with ammoniacal silver hydroxide. Silver-staining material occurs in the form of minute dots on the surface. In many places these are enlarging and fusing to form irregular blotches. The cell areas are clearly marked out.

Fig. 11. The same field as fig. 10 at a slightly lower focus showing in optical transverse section the silver-staining filaments in the pore canals. The cell boundaries can be seen in places but are not so distinct.

Fig. 12. Surface view of the new ventral cuticle in 7-day-old pupa treated with ammoniacal silver hydroxide. Polyphenol layer almost complete over the cell areas; mostly absent along the intervening regions. This silver-staining layer is absent also from the pits which appear as oval white spots.

## PLATE II

Magnifications as in Plate I, all taken with 4-mm. objective, except fig. 2, taken with 2-mm. objective, and fig. 4 taken with 16-mm. objective.

Fig. 1. Surface view of the new ventral cuticle in late 7-day-old pupa treated with ammoniacal silver hydroxide. Polygonal areas corresponding with individual cells are visible here and there, but for the most part the polyphenol layer is covered and only occasional rounded spots stain. (The punctate areas are the openings of the male pit glands.)

Fig. 2. Detail of the same showing the punctate staining of the pore canals around the silver-staining spots.

Fig. 3. Adult in act of emerging from the pupa treated with ammoniacal silver hydroxide. Only a few scattered spots stain. To the right is presumably a mechanical abrasion of the covering wax layer.

Fig. 4. Newly moulted adult, treated 5 minutes in boiling chloroform before immersion in ammoniacal silver hydroxide. Low power view of ventral abdominal integument from inside. The polyphenol layer gives a continuous dark stain except in the pits, which show as white spots with black centres (the male pit glands). The black convoluted contents of the dermal glands are conspicuous.

Fig. 5. Newly moulted adult female treated as fig. 4. Surface view of ventral abdominal integument. The white spots are the pits. The black spots are the contents of the distended dermal glands.

Fig. 6. The same. Dorsal abdominal integument. The black objects are the silver-staining contents of the dermal glands. The two white spots are companiform sensilla.

Fig. 7. Recently moulted adult treated as fig. 4. Exposure of the polyphenol layer is incomplete even after chloroform extraction.

Fig. 8. Adult male 30 minutes after moulting, treated as fig. 4. Above and to the right the cement layer has been discharged; there is a finely granular superficial staining, the dermal glands are empty, and the polyphenol layer stains only in a few rounded spots. Below and to the left the staining of the polyphenol layer is almost continuous; the dermal glands, still filled with secretion, can be faintly seen through the cuticle.

Fig. 9. A preparation at the same stage similar to Fig. 8. Above and to the left the finely granular cement layer can be seen, with darker lines along the intercellular boundaries; the polyphenol layer is exposed only in rounded spots. Below and to the right there is continuous staining of the polyphenol layer (except in the pits) and the distended dermal glands are faintly visible below.

Fig. 10. Adult female 1 hour after moulting, treated as fig. 4. Dermal glands emptied. No staining of polyphenol layer. Superficial silver staining of the granular cement layer, particularly along the intercellular boundaries.
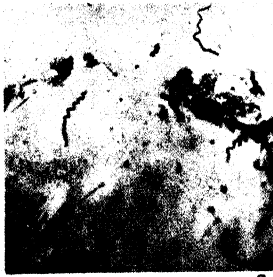
Fig. 11. Newly moulted adult; ventral cuticle gently rubbed with alumina and immersed directly in ammoniacal silver hydroxide. Almost continuous staining of polyphenol layer except in the pits and here and there along the intercellular boundaries.

Fig. 12. Adult 6 hours after moulting; same treatment as fig. 11. Silver staining in the form of small spots.
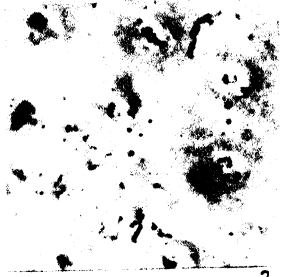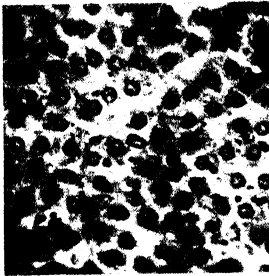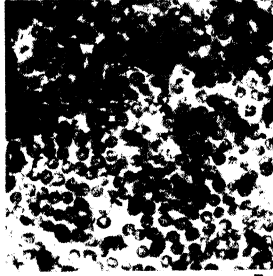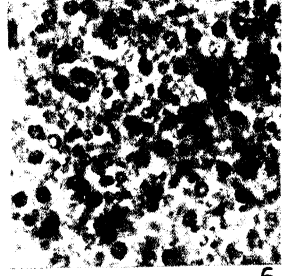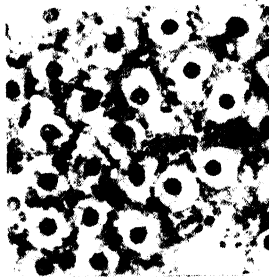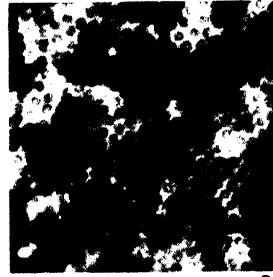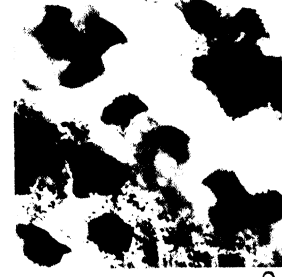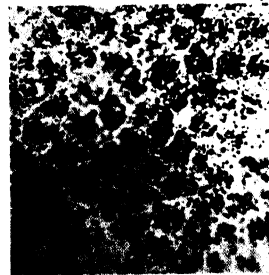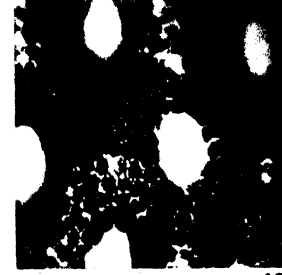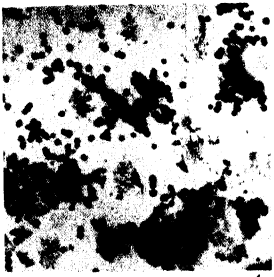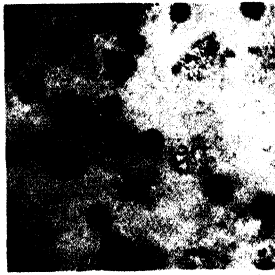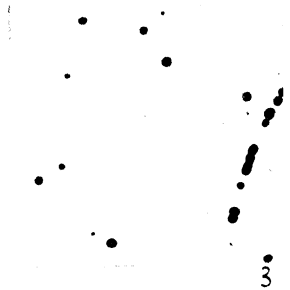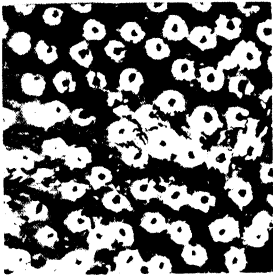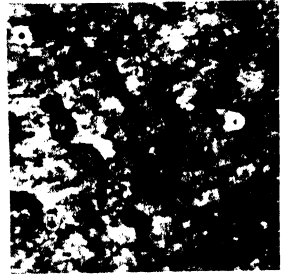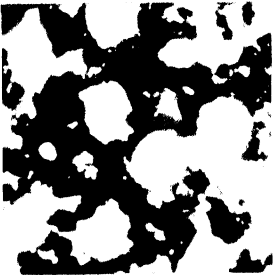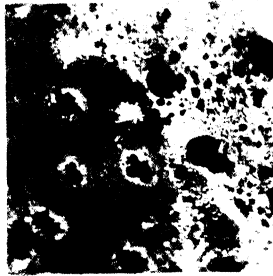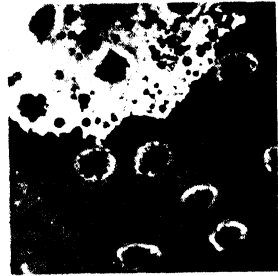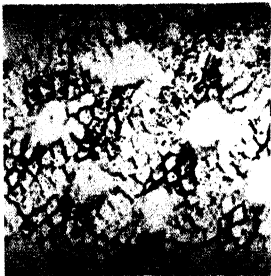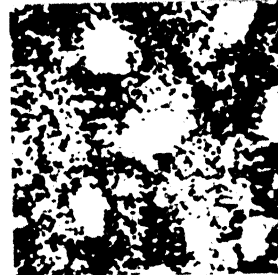
# A Study of the Cytoplasmic Components during the Gametogenesis of *Bos taurus*

BY

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**With one Plate and fourteen Text-figures**

## INTRODUCTION

THIS paper is an account of the Golgi material and mitochondria of the germ-cells, and of spermateleosis, of domestic cattle (*Bos taurus*). Zlotnik (1943) described a nuclear ring present in the spermatids and spermatozoa of the bull, but so far as the writers are aware there is no previous account of the cytoplasmic components during the gametogenesis of this animal. Much of the literature on the cytoplasmic components of the germ-cells of mammals has been discussed recently by Gresson and Zlotnik (1945) and by Zlotnik (1948).

## MATERIAL AND METHODS

The ovaries of three heifers, aged from 9 to 11 months, were fixed in Champy-Kull, Kolatchev, and Aoyama. For the examination of the Golgi material pieces of ovaries fixed according to the method of Kolatchev were the most satisfactory. Small portions of the ovaries of two cows, aged 4 years, were fixed in Susa and Bouin.

For the study of the male germ-cells pieces of the testes of three bulls of varying ages were fixed in Flemming (without acetic), Kolatchev, and Aoyama. The sections fixed in Kolatchev were stained with acid fuchsin or with haematoxylin.

In all cases the animals were killed and small pieces of tissue placed in the fixing fluid as speedily as possible. Sections were subsequently cut at $5\,\mu$ and at $8\,\mu$ in thickness.

## OBSERVATIONS

### *Oogenesis*

All stages of the development of the primary oocyte were represented in the material examined, and in addition oogonia were visible in some of the sections. The history of the oocyte is of the usual mammalian type. The older primary oocytes possess a well-marked zona radiata. Follicles in process of rupture were not observed.

*The Golgi Material*

*Oogonia.* The Golgi material is a compact mass which occupies a juxta-nuclear position. Owing to its small size and compact nature it was not possible to determine its structure with certainty, but it is probable that it is composed of Golgi elements arranged in a manner similar to those of the young primary oocyte.

*Oocytes.* The Golgi material of oocytes surrounded by a few elongate follicle-cells is situated at one side of the nucleus (Pl. 1, fig. 1). Careful study of this material indicates that, in the oocyte of the cow at least, it is not in the form of a network, but consists of spherical and rod-shaped bodies. In oocytes surrounded by a single layer of columnar follicle-cells the position of the Golgi material varies. In some oocytes it is still localized at one side of the nucleus; in some of these cells deeply impregnated granules and rods are present in the cytoplasm, particularly in the neighbourhood of the nucleus. The Golgi material of a few cells consists of two parts, usually situated at opposite sides of the nucleus, and of rods and granules scattered through the cytoplasm (Pl. 1, fig. 2). The sequence of events is as follows. When the follicle-cells become columnar, the juxta-nuclear Golgi material spreads out over part of the nucleus. Very soon granules and small rod-shaped Golgi elements leave the localized mass and are distributed through the cell. At a slightly later stage large masses of Golgi substance, as well as small bodies, move out through the cytoplasm.

Two large masses of Golgi material are still present in some of the cells surrounded by two layers of follicle-cells. They rapidly break up into smaller bodies, and shortly after a third layer of follicle-cells is formed the Golgi substance consists of small granules in the central cytoplasm, and of large bodies situated in the peripheral region of the cell (Pl. 1, fig. 3).

With the further growth of the follicle, small spaces appear within the follicular epithelium and the zona radiata is formed. Oocytes at this stage are of two types as regards the arrangement of the Golgi material and the number of fat globules present. In one type, globules of fat are relatively few, and the Golgi material is composed of comparatively large rods and granules lying at the periphery of the cell, and of very fine granules scattered through-out the cytoplasm. In the second kind of oocyte the Golgi bodies in the peripheral region are very much smaller (Pl. 1, fig. 4). In these cells fat globules are much more numerous than in oocytes of the first type, and the majority are located in loose clumps which may extend from the periphery to the central region. The number of fat globules present and the form of the peripheral Golgi bodies closely resembles the condition of the oocytes of Graafian follicles (Pl. 1, fig. 5). It is concluded, therefore, that these oocytes are at a later stage of development than those possessing comparatively few fat globules and larger Golgi bodies.

*Follicle-cells.* The Golgi material of the young elongate follicle-cells is a small compact mass at one side of the nucleus. When the cells become columnar, the Golgi substance is sometimes situated at the pole of the nucleus

next to the oocyte, but in many cells it is still located at one side of the nucleus (Pl. 1, fig. 2). At a slightly later stage, the Golgi material of all the cells lies between the nucleus and the oocyte. In older follicles, which are still composed of a single layer of cells, the Golgi substance is less compact and is visibly composed of rods and granules which surround a clear area.

In follicles consisting of two layers, the Golgi material is similar in appearance to that of the preceding stage, but its position in relation to the nucleus varies. The Golgi material of the majority of cells in the layer adjacent to the oocyte is at the pole of the nucleus next to the oocyte, in a few cells it is at the side of the nucleus, and in others it is situated in the end of the cell directed away from the oocyte. In all the cells of the outer layer the Golgi material is located at the pole of the nucleus directed towards the oocyte. In follicles composed of three layers, the Golgi substance of most of the cells of the layer nearest to the oocyte is localized at the pole opposite to the oocyte, but in a few cells it is still situated at the side of the nucleus. In the other two layers, the Golgi material is directed towards the oocyte.

In follicles composed of several layers, and in Graafian follicles, the Golgi material of the majority of the cells of the two layers nearest to the oocyte are directed away from the oocyte; in the cells of the other layers it is directed towards the oocyte.

The Golgi material of the cells of older follicles is frequently less compact than in very young follicles and is often visibly composed of granules and rods. In many of the larger follicles, bodies, which are probably granules of secretion, are present in contact with the Golgi substance and in the surrounding cytoplasm.

### Mitochondria

*Oogonia.* The mitochondria are very fine granules which surround one pole of the nucleus. They are very faintly stained and, owing to their small size, are often difficult to detect.

*Oocytes.* The mitochondria of oocytes surrounded by a single layer of elongate follicle-cells are, like those of the oogonia, minute granules which are only clearly visible in certain parts of the sections. They surround one pole of the nucleus and extend to the equatorial region. At a slightly later stage, when the follicle-cells are broader but are still arranged with their long axes parallel to the oocyte, the mitochondria begin to spread out through the cytoplasm. When most of the follicle-cells have become columnar, the mitochondria are scattered in groups throughout the cell; the majority have increased in size and many now stain deeply.

In oocytes surrounded by two layers of follicle-cells, the mitochondria are more numerous and the majority are larger than during the preceding stage. They are absent from the vicinity of the nucleus, but are distributed fairly evenly throughout the rest of the cell.

The mitochondria of oocytes situated in follicles consisting of several layers are arranged in a broad peripheral zone. They vary considerably in size;

many are large granules or small spheres, while others are in the form of fine granules. When the small cavities within the follicular epithelium run together to form the antrum, the mitochondria begin to move away from the periphery, so that groups extend from the peripheral layer towards the central cytoplasm (Pl. 1, fig. 6).

In oocytes of Graafian follicles, the mitochondria form a large mass which lies in the central cytoplasm and is in contact with one pole of the nucleus. A few small groups of granules are scattered through the cell and in most cases surround fat globules (Pl. 1, fig. 7).

*Follicle-cells.* The mitochondria were badly preserved and were often invisible; in a number of cells, however, fine granular mitochondria were recognized. In the young elongate follicle-cells, the mitochondria are at one side of the nucleus. At a later stage they are situated between the nucleus and the oocyte. When the follicle consists of several layers, the mitochondria of the cells of the first layer lie in the part of the cell which is directed away from the oocyte, while those of the cells of the other layers are in the cytoplasm nearest to the oocyte. In the older follicles, the cytoplasm of many cells, except those in contact with the oocyte, is drawn out into a long process which extends towards the oocyte and contains mitochondria and frequently secretory granules.

## Yolk

Small globules are visible in young oocytes surrounded by a single layer of columnar follicle-cells. Since these are deeply blackened in Kolatchev preparations and are represented by clear spaces in material fixed in Susa fixative, they are identified as fat globules. In the older oocytes, the globules increase in size and number, and in the oocytes of Graafian follicles several large globules, usually surrounded by small Golgi elements and by mitochondria, are present, chiefly towards the periphery. The reduction in the size of the Golgi bodies, at the time when there is a marked increase in the amount of fat, suggests that the peripheral Golgi material may play some part in the formation of the globules.

The young oocytes at first contain a single nucleolus and a number of small bodies which stain in a similar manner. Later, more than one nucleolus is present and the small bodies are more numerous. As the small bodies are often in contact with nucleoli, it is possible that they originate as buds from the latter. The presence of bodies of similar size and staining properties in the vicinity of the nuclear membrane and within the cytoplasm of oocytes surrounded by one or more layers of follicle-cells suggests that the buds are extruded from the nucleus. If these bodies are nucleolar extrusions, it is probable that they contribute to the nutritive material of the egg. As they stain in a similar manner to the mitochondria their history could not be followed in the material available, even in oocytes fixed in Bouin's fluid in which the mitochondria are imperfectly preserved. Cytoplasmic bodies were not present in late oocytes fixed in Susa fixative; this may indicate that the

nucleolar extrusions are used up in the formation of nutritive material. Unfortunately, young oocytes were not present in the ovaries fixed in Susa.

### Spermatogenesis

The history of the cytoplasmic components during the spermatogenesis of the bull in general resembles the history of these structures in other mammals investigated by the writers (Gresson, 1942; Gresson and Zlotnik, 1945). It is unnecessary, therefore, to give a lengthy account of the stages of spermatogenesis.

*Spermatogonia.* The Golgi material is made up of rods and granules which lie at one pole of the nucleus (Text-fig. 1). Each element consists of a deeply impregnated cortex and a lightly impregnated central region. The mitochondria surround the nucleus and are most numerous in the immediate vicinity of the Golgi material.

*Spermatocytes.* In the resting primary spermatocytes the Golgi material is localized, and granular mitochondria are scattered round the nucleus. A body which blackens with osmium tetroxide is visible close to the Golgi material of many of the primary spermatocytes (Kolatchev preparations); bodies of approximately the same size, but somewhat lighter in colour, are often present elsewhere in the cytoplasm (Text-fig. 2). Similar bodies are present in Flemming preparations; they are identified as accessory bodies.

During the prophase of the first spermatocyte division, the Golgi material becomes less compact. Many of the Golgi elements are larger than those of the spermatogonia and younger spermatocytes, and the cortical and central regions are more clearly visible (Text-fig. 3). The mitochondria become scattered through the cytoplasm, and remain in this condition throughout the subsequent stages of division (Text-fig. 4). During the metaphase and anaphase the Golgi material consists of two widely separated groups of elements lying towards opposite poles of the cell. Spermatocytes at late prophase were not identified in Kolatchev and Aoyama preparations, but observations indicate that the separation of the Golgi elements into two groups takes place during the late prophase or early metaphase. Accessory bodies are present in the cytoplasm during the stages of division (Text-figs. 3 and 4).

*Spermateleosis.* The Golgi material of the young spermatid is localized at one pole of the nucleus; the mitochondria are scattered through the cytoplasm but are slightly more numerous in the neighbourhood of the Golgi material than elsewhere in the cell. An accessory body is often visible close to the Golgi material (Text-fig. 5). At a later stage an archoplasmic vacuole, containing an archoplasmic granule, is present. The acrosome arises from these structures (Text-figs. 6–10). When the Golgi material migrates to the posterior pole of the nucleus the elements of which it is composed become closely clumped together (Text-figs. 7 and 8). It remains for some time in contact with the posterior pole of the nucleus. Later, argentophilic and osmiophilic granules appear in the vicinity of the posterior part of the nucleus
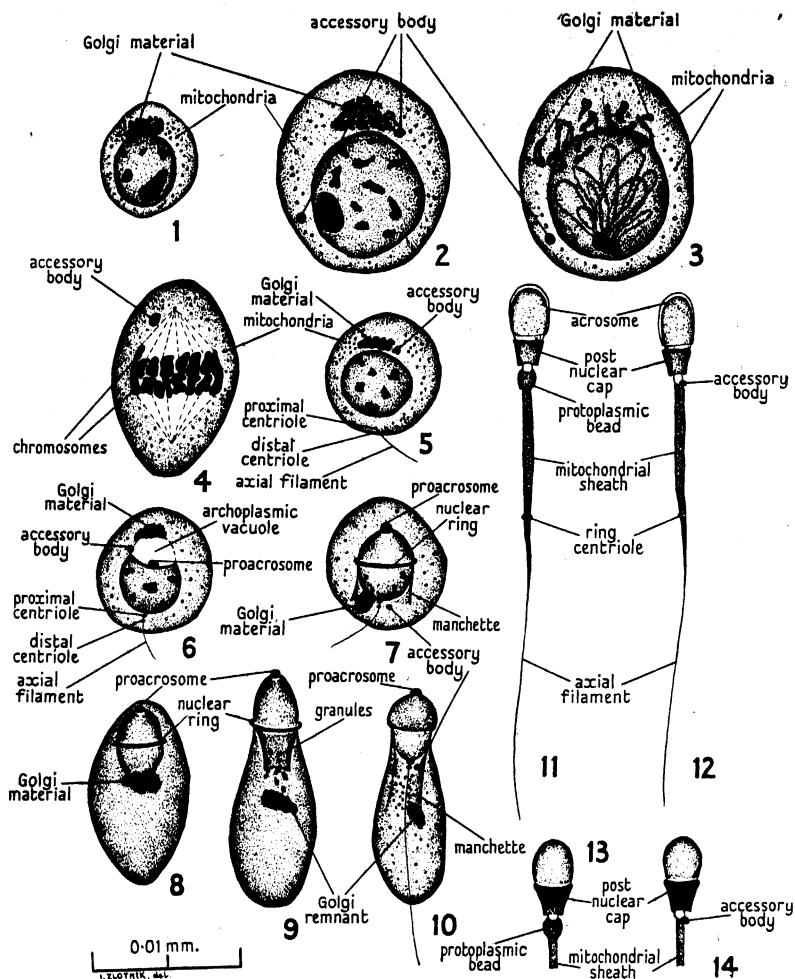
Text-figures 1, 2, 3, 5, 6, 7, and 10 from Kolatchev preparations. Text-figures 4, 11, and 12 from Flemming preparations. Text-figures 8, 9, 13, and 14 from Aoyama preparations.

TEXT-FIG. 1. Spermatogonium.
TEXT-FIG. 2. Primary spermatocyte.
TEXT-FIG. 3. Primary spermatocyte—early prophase.
TEXT-FIG. 4. Primary spermatocyte—early anaphase.
TEXT-FIG. 5. Young spermatid.
TEXT-FIGS. 6–8. Spermatids.
TEXT-FIGS. 9 and 10. Late spermatids.
TEXT-FIG. 11. Spermatozoon.
TEXT-FIG. 12. Spermatozoon showing accessory body; protoplasmic bead not shown.
TEXT-FIG. 13. Spermatozoon.
TEXT-FIG. 14. Spermatozoon showing accessory body; protoplasmic bead not shown.

(Text-fig. 9). As some of these granules are often in contact with the Golgi material it is probable that they originate from it. Two centrioles were identified. The proximal one is large and soon comes into contact with the posterior pole of the nucleus; the distal centriole passes to the end of the middle-piece where it persists as the ring centriole (Text-figs. 11 and 12). The nuclear-ring arises on the rim of the nuclear depression produced by the archoplasmic vacuole (Text-figs. 6–9). The manchette marks out the cytoplasm which is included in the middle-piece of the spermatozoon (Text-fig. 10).

The mitochondrial sheath of the middle-piece of the spermatozoon is formed by the majority of the mitochondria of the late spermatid which become concentrated inside the manchette (Text-figs. 10–12). The post-nuclear cap is deeply impregnated in silver preparations. A protoplasmic bead, with argentophilic granules on its surface, is situated at the proximal end of the middle-piece (Text-figs. 11 and 13). An accessory body is present in the neck region of the spermatozoon (Text-figs. 12 and 14).

## DISCUSSION

The present work indicates that the localized Golgi material of the oogonia, oocytes, spermatocytes, and spermatids of domestic cattle is not a network; this agrees with comparatively recent observations on the germ-cells of other mammals (Gresson, 1940 and 1942; Gresson and Zlotnik, 1945; Zlotnik, 1948). The method of dispersal of the Golgi material of the young oocyte varies in different animals. The young oocyte of the cow appears to differ from those of the other mammals studied in that, prior to its dispersal, the Golgi material separates into two masses which come to lie at opposite sides of the nucleus.

Henneguy (1926) states that in follicles of the guinea-pig consisting of a single layer, the Golgi material is oriented towards the oocyte. In follicles of several layers, the position of the Golgi material varies, and in the discus proligerus it lies between the nucleus and the follicular cavity. He believes that the cells at first secrete a substance which is passed into the oocyte, and later take part in the formation of the follicular liquid. The location of the Golgi material in the follicle-cells of the mouse led Gresson (1933) to support Henneguy's suggestion. More recently it has been shown that the Golgi substance of the follicle-cells of other mammals undergoes a change of polarity correlated with the growth of the follicle. Beams and King (1938) state that, in the guinea-pig, the Golgi material 'is generally polarized towards the egg or towards the follicular cavity—a condition which is taken to indicate that the more physiologically active pole of the cell is marked by the position of the Golgi apparatus'. The work of Aykroyd (1938) on the human oocyte, and of Zlotnik (1948) on the oocytes of the dog, the cat, and the rabbit, demonstrates that the Golgi material of many of the cells in the older follicles undergoes a change of polarity and is often situated in relation to the accumulation of the follicular liquid.

If the location of the Golgi material is an indication of functional polarity, then its change of position in the follicle-cells of the cow indicates that these cells at first form a secretion which is passed into the oocyte, and later produce a substance which accumulates in the follicular cavity. The presence of secretory granules in the vicinity of the Golgi material of cells in late follicles supports this view, but the migration of granules into the follicular cavity was not observed. In certain mammals the passage of secretory granules from the follicle-cells into the oocyte has been recorded (Zlotnik, 1948). The mitochondria undergo changes of position which are probably correlated with the functional polarity of the cell.

The behaviour of the mitochondria of the oocyte of the cow is in general similar to that of the mitochondria of other mammals. In oocytes of Graafian follicles, however, the majority form a large mass in the central cytoplasm, while in the other mammals studied the mitochondria are fairly evenly scattered throughout the cytoplasm, or else there is a peripheral concentration in addition to small groups situated in the central region of the cell.

As regards yolk-formation, there is no direct evidence that the cytoplasmic components of the oocyte of the cow participate in the process. Yolk globules are frequently surrounded by Golgi bodies and by mitochondria, and it may be that these structures are concerned with the elaboration of the globules. Extrusion of nucleolar material to the cytoplasm takes place, and possibly adds to the nutritive material of the ovum. The literature on yolk-formation in the mammalian egg is conflicting; Zlotnik (1948) has recently discussed this process in some mammals.

The more recent work on the cytoplasmic components of the male germ-cells of mammals is reviewed by the writers in a previous paper (Gresson and Zlotnik, 1945). In the present contribution, therefore, certain aspects of spermatogenesis only are discussed.

The time at which the Golgi material of the spermatocyte is dispersed varies in different mammals. In the bull the Golgi elements do not scatter through the cytoplasm, as in some animals, but separate into two groups during the late prophase or early metaphase.

Wadsedalek (1914) records the presence of a 'chromatoid body' in the germ-cells of the bull. The writers believe that this structure is an accessory body. Accessory bodies are present in the spermatocytes and spermatids of other mammals, and are believed by Gresson and Zlotnik (1945) to originate from the localized Golgi material and to give rise to the Golgi substance of the spermatozoon.

The argentophilic and osmiophilic granules present at the posterior end of the nucleus of the late spermatid most probably originate from the Golgi material, and some of them, at least, form the granules of the protoplasmic bead. It is possible that some of the granules may take part in the formation of the post-nuclear cap.

Spermatozoa within the seminiferous tubules of the bull possess a protoplasmic bead. This agrees with previous observations of Gresson and Zlotnik

(1945). Gatenby and Collery (1943) state that two beads are present on the spermatozoon of the dog and the guinea-pig, and that the lower one (protoplasmic bead) is not present on sperms within the testis but only on sperms from the epididymis. The writers found that the protoplasmic bead of the other mammals examined by them is eliminated after the spermatozoa enter the epididymis. Smears from the epididymis of the bull were not examined.

The present work confirms previous observations of Gresson and Zlotnik (1945) that the distal centriole does not divide, and that the manchette enters into the formation of the middle-piece of the spermatozoon.

We wish to express our thanks to Professor James Ritchie for research facilities, and to Mr. J. R. Fant for taking the photomicrographs.

## Summary

### Oogenesis

1. The Golgi material of the oogonium and the early primary oocyte consists of spherical and rod-shaped bodies situated at one side of the nucleus. In older oocytes it is present as rods and granules distributed through the cell.

2. Granular mitochondria surround the nucleus of the oogonium and the early primary oocyte. In older oocytes they are scattered in groups through the cytoplasm. Later, they are arranged in a broad peripheral zone. In oocytes of Graafian follicles the majority of the mitochondria are situated in the central cytoplasm.

3. Small fat globules are present in young oocytes. In older oocytes the globules increase in size and number. There is a reduction in the size of the Golgi elements at the time when there is a marked increase in the amount of fat; this suggests that the Golgi material plays a part in the formation of the globules. Nucleolar material appears to be passed into the cytoplasm; it may contribute to the formation of nutritive material.

4. The position of the Golgi material of the follicle-cells suggests that the cells at first form a secretion which is passed into the oocyte, and later a secretion which enters the follicular cavity. Secretory granules are visible in many of the cells of late follicles. The position of the mitochondria appears to be correlated with the functional polarity of the cell.

### Spermatogenesis

1. The Golgi material of the spermatogonium, resting spermatocyte, and early spermatid consists of rods and granules situated at one side of the nucleus. The Golgi elements separate into two groups during nuclear division. Argentophilic and osmiophilic granules present at the posterior pole of the nucleus of the late spermatid probably originate from the Golgi material. The Golgi remnant is eliminated with the residual cytoplasm.

2. Accessory bodies are present in primary spermatocytes and in spermatids. An accessory body is present in the neck region of the spermatozoon.

3. The mitochondria are granular; their behaviour during spermatogenesis is described.

4. The formation of the acrosome and the nuclear-ring is briefly described.

5. The distal centriole passes undivided to the posterior end of the middle-piece. The manchette marks out the cytoplasm which is included in the middle-piece.

6. The protoplasmic bead, present at the anterior end of the middle-piece, is formed while the spermatozoon is within the testis.

## REFERENCES

AYKROYD, O. E., 1938. Z. Zellforsch. mikr. Anat., **27**, 691.

BEAMS, H. W., and KING, R. L., 1938. Cytologia, **8**, 353.

GATENBY, J. B., and COLLERY, L., 1943. Nature, **151**, 253.

GRESSON, R. A. R., 1933. Quart. J. micr. Sci., **75**, 679.

—— 1940. Ibid., **81**, 569.

—— 1942. Proc. Roy. Soc. Edin. B, **61**, 197.

—— and ZLOTNIK, I., 1945. Ibid., **62**, 137.

HENNEGUY, L. F., 1926. C.R. Soc. Biol., **94**, 674.

WADSEDALEK, J. E., 1914. Biol. Bull., **27**, 295.

ZLOTNIK, I., 1943. Nature, **151**, 670.

——1948. Proc. Roy. Soc. Edin. B (in press).

## DESCRIPTION OF PLATE I

Photomicrographs. Figs. 1–5 from Kolatchev preparations. Figs. 6 and 7 from Champy-Kull preparations.

Fig. 1. Young oocyte showing localized Golgi material. × 960.

Fig. 2. Young oocyte; the Golgi material has separated into two masses situated at opposite sides of the nucleus. The Golgi material of some of the follicle-cells is shown. × 960.

Fig. 3. Oocyte showing scattered Golgi bodies and a few fat globules. × 480.

Fig. 4. Late oocyte showing fat globules surrounded by Golgi bodies. Golgi bodies are also scattered through the cytoplasm. × 480.

Fig. 5. Oocyte of Graafian follicle showing fat globules and Golgi bodies. × 480.

Fig. 6. Late oocyte to show mitochondria. × 480.

Fig. 7. Oocyte in Graafian follicle to show mitochondria. × 480.
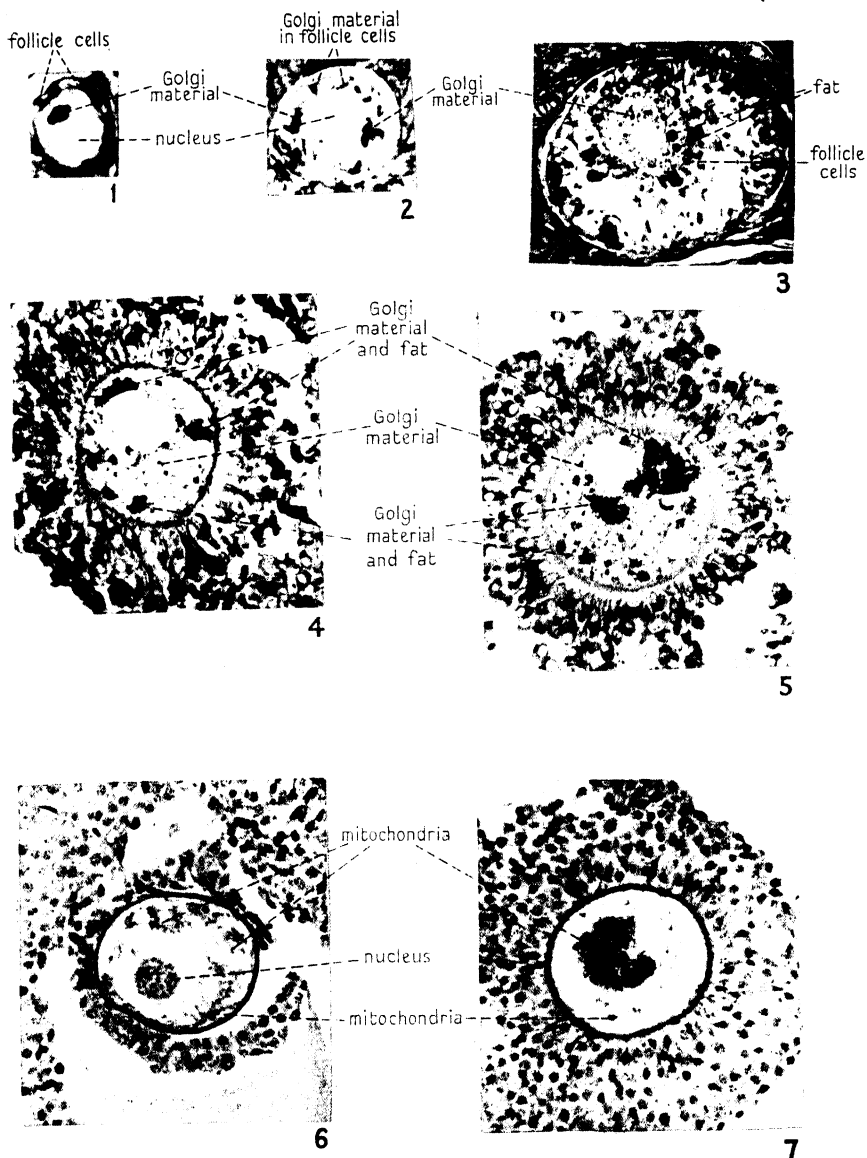
R. A. R. GRESSON AND I. ZLOTNIK—PLATE I

# An Easily Controlled Method for Staining Mitochondria

BY

A. J. CAIN

*(From the Department of Zoology and Comparative Anatomy, Oxford)*

THE great disadvantages of the standard staining methods for mitochondria are that they are very complicated and cannot be applied to different tissues until suitable times for the action of the various stains have been worked out by a long process of trial and error.

The following method is easy to control and apply. Destaining and restaining are quickly carried out, times may be worked out on a single section, and, as each reagent is used for a single purpose (not, for example, as a simultaneous differentiator and stain), each stage may be controlled separately. Mitochondria are red, cytoplasm is pale blue or colourless, nuclei are medium blue, and plasmosomes (nucleoli) pink or red.

1. Fix small pieces in Helly's fluid for 6 hours.
2. Postchrome for 48 hours at 37° C. in a saturated aqueous solution of potassium dichromate.
3. Wash overnight in running water.
4. Embed in paraffin wax and cut sections of a suitable thickness (about 3 $\mu$). Bring the sections down to water, treating with iodine ($\frac{1}{2}$ in 70 per cent. alcohol) and then sodium thiosulphate (5 per cent. aqueous solution) on the way.
5. Dry a slide except where the sections are, flood with acid fuchsine in aniline-water, and heat gently until 'steaming', in order to overstain, exactly as for Altmann's technique. (For detailed instructions see Baker, 1945, p. 190.)
6. Wash off the acid fuchsine with distilled water, observe the section under the high power of the microscope, and irrigate with an alkaline solution. One drop of saturated aqueous sodium carbonate solution in 10 c.c. of distilled water gives a fairly rapid differentiation, taking 30 sec. to 1$\frac{1}{2}$ minutes.
7. To stop differentiation and brighten the acid fuchsine, dip the slide into 1 per cent. hydrochloric acid. For the criteria of differentiation see below. If brightening is undesirable because extraction of the dye from the cytoplasm is difficult, wash in distilled water instead of dipping into the acid.
8. When the slide has been correctly differentiated, wash it in distilled water, then counterstain progressively in a $\frac{1}{2}$ per cent. aqueous solution of water-soluble methyl blue. The stain 'Soluble Blue' (not 'soluble blue, crystals AS') sold by B.D.H. is suitable. For a discussion of methyl blues see below. Wash off the stain with distilled water, dip in 1 per cent.

acid for 3 seconds only, wash with distilled water, go through the alcohols (in no great hurry), and mount in Canada balsam.

After the acid fuchsine has been applied, the section is brilliant red throughout, though it may be possible to see that the mitochondria are darker than the rest of the cytoplasm. Differentiation should proceed until the mitochondria are dark red against pink cytoplasm. Some cells (e.g. nephridial cells of the leech *Glossiphonia complanata* (L.)) will give up all the acid fuchsine from the cytoplasm before the mitochondria begin to pale. Others (e.g. intestinal epithelium of the mouse) retain a certain quantity, but it is easily masked by the methyl blue. For such cells as the former, comparatively thick sections (5–10 $\mu$) may be used, but for the latter they should be thin (3 or 2 $\mu$). If differentiation is carried too far, the section can be restained in acid fuchsine.

When the staining and differentiation of the acid fuchsine are satisfactory, the nucleus and cytoplasm are counterstained with a methyl blue dye. This dye, being acid, does not interfere with the differentiation of the acid fuchsine. Many basic dyes or dye-lakes can be used to differentiate acid dyes. For example, Kull (1914) used toluidine blue to differentiate acid fuchsine, but as the blue had overstained the section before completing differentiation, he used aurantia to differentiate the differentiator, thereby producing his well-known trichrome method.

The trouble with such procedures is that the times of suitable differentiation and suitable staining by a combined stain and differentiator are unlikely to be the same, and it is difficult to control a differentiation proceeding in a coloured solution. Trichrome methods give beautiful results when properly carried out, but dichrome methods are perfectly satisfactory for almost all purposes, and with a colourless differentiator are far easier to control. Quite good preparations can be made from mouse small-intestine epithelium (and probably from other tissues) by overstaining in acid fuchsine, and staining and differentiating simultaneously with Ehrlich's haematoxylin, tap-water (which is not sufficiently alkaline to disturb the acid fuchsine) being used for blueing. A suitable time in the haematoxylin for 3 $\mu$ sections of this tissue is 7 minutes. But the cytoplasm is not very clear and the contrast with the red in the mitochondria is rather poor. Methyl green (a basic dye) may be used as in Bensley's method (1911) but it is exceedingly rapidly removed by alcohol and very sensitive to acid balsam. Methyl blue (water soluble) when acting as an acid dye is superior because differentiation of the acid fuchsine can be carried out separately and it will not interfere, it can be made to stain nuclei well, it gives good colour-contrast with the mitochondria, and it is not sensitive to alcohols and acid balsam. (It is removed by strong alcohol, but, as with acid fuchsine, only after periods of some hours.) If overstaining occurs it can be removed, but much more rapidly, by the same differentiator as was used for acid fuchsine, followed by 70 per cent. alcohol.

The group of dyes usually called methyl blue (excluding methyl blue SS) are closely related acid triphenylmethane dyes. They can behave as acid or as

basic dyes according to the circumstances of their preparation and the pH of the media in which they are dissolved. In alkaline solutions they are dull blue, rather slowly staining, acid dyes, rather easily removed by alcohol. In acid solution they are intensely blue basic dyes fast to alcohol. When dissolved in distilled water they may behave as acid or as alkaline dyes according to the circumstances of preparation. Various samples were examined, and it was found that those that are sold in the form of crystals with a reddish-bronze lustre and give intensely reddish-blue solutions in distilled water behaved as basic dyes, and those sold as dark-blue powders giving a less intense and rather dull-blue solution in distilled water behaved as acid dyes. B.D.H. Soluble Blue is an example of this class. An acid dye is essential to avoid further differentiation of the acid fuchsine, which is rapidly removed by the basic methyl blue dyes. Use of a basic methyl blue in an alkaline solution cannot be recommended because, if the solution is sufficiently alkaline for the dye to be acidic, then it will itself differentiate the acid fuchsine. It is therefore essential to use an acid dye in distilled water. As the colour produced by it is purplish and rather feeble, the dye can be made much more intense and less purple in tone by dipping the slide into 1 per cent. hydrochloric acid for a few seconds only. If the slide is left in it for longer, the methyl blue, now basic, will attack the acid fuchsine. Intensification is complete in 2 or 3 seconds, and the slide is immediately removed and freed from acid by washing with distilled water.

Methyl blues are fairly readily removed by 70 per cent. alcohol when in the alkalinized state, that is, when behaving as acid dyes. If a valuable slide has been overstained with methyl blue, it can be destained by a minute's treatment with sodium carbonate solution followed by a quarter of an hour in 70 per cent. alcohol, and may then be restained with acid fuchsine and methyl blue.

The method described has given satisfactory results with the intestine, liver, and kidney of the mouse, and nephridial cells, muscle-fibres, and gut epithelium of the leech *Glossiphonia*. Kidney should be cut if possible at $2 \mu$, as the mitochondria are clustered so thickly together. Suitable times for differentiation and counterstaining are best worked out on the first slide of each batch, and then applied to the rest. The time for methyl blue varies greatly with the thickness of the section, thicker sections requiring much less staining than thinner ones. $3 \mu$ sections of mouse intestine required 1 minute, $10 \mu$ sections only 30 seconds.

### SUMMARY

A method for staining mitochondria is described in which sections are overstained with acid fuchsine, differentiated in sodium carbonate solution, and counterstained with a methyl blue acting as an acid dye.

### REFERENCES

BAKER, J. R., 1945. *Cytological Technique*, 2nd edition. London (Methuen).
BENSLEY, R. R., 1911. Amer. J. Anat., **12**, 297.
KULL, H., 1914. Anat. Anz., **45**, 153.

# A New Method for Oblique Microscopical Illumination

BY

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With two Text-figures

THE purpose of the technique described in this paper is to provide the easiest possible means of controlling the direction and degree of obliquity of the light used to illuminate microscopical objects.

The method is applicable when striations or other regularly-repeated markings on objects are situated so close together that, with axial illumination, the diffraction-spectrum they produce lies partly or wholly outside the back lens of the objective. A clear image can in these circumstances only be produced by throwing the direct light to one side of the back lens, and thus making room for the spectrum on the other side. Methods for achieving this object are familiar to microscopists. Advantages are claimed for the new method described in this paper. The intention is to allow the microscope to be used at its ultimate resolving-power with the greatest possible convenience and effectiveness. The method is likely to be useful in the testing of high-power objectives and in the study of the minutest markings on the shells of certain diatoms.

The basis of the method is that the *objective* of the microscope is used as an *eyepiece* through which the direction and degree of obliquity of the light are examined while the mirror is tilted in various ways. The condenser has to be in a special position in order to enable the objective to be used in this way.

No special apparatus is required. The source of light must be rather bright: an ordinary 100-watt filament-lamp with 'pearl' glass is very convenient. ('Opal' glass does not let through enough light.) Two stops with circular apertures are needed, with some easy means of changing from one to the other. One stop should have an aperture about $3\frac{1}{2}$ or 4 mm. in diameter, the other about 15 mm. These will be called respectively the small and large stops. The stop in use must be placed immediately in front of the lamp. (An iris diaphragm can be used instead, but is rather less convenient.) The lamp must be arranged in such a way that a line drawn from the centre of the mirror through the centre of the stop would pass through the brightest part of the lamp. It is also desirable that this line should be approximately at right angles to the optical axis of the microscope. A stainless steel mirror is preferable to a glass one, because it gives only a single image, but it is by no means a necessity for the successful working of the method. An oil-immersion condenser of wide aperture (such as Watson's Holoscopic) is required; it must be accurately

centred and the iris diaphragm below it must be kept wide open. An oil-immersion objective of high aperture is of course necessary. The eyepiece used should be fairly powerful (I use a Watson's Holoscopic ×14). It is useful to have also a short cork that fits the top of the draw-tube, with a hole about 8 mm. in diameter bored through the middle of it. The use of this will be mentioned at the appropriate place.

The method for showing the striations of the shell of *Amphipleura pellucida* will now be described as an example. As is well known, this elongated diatom is transversely striated, the lines being exceedingly regular and closer together than the shortest wave-length of visible light. It is best to have the diatoms mounted in hyrax. Choose a specimen that lies somewhat apart from others so as to avoid complications arising from the appearance of more than one spectrum. (The presence of part of another diatom near the edge of the field of view is not harmful.) It does not matter in what direction the chosen diatom is orientated. In this description I shall suppose, for simplicity, that it happens to lie with its long axis in the 'north and south' direction. The striations, which will at first be invisible, will be directed east–west.

Place the small stop in front of the lamp and by means of the condenser focus the image of the stop so that it is clearly seen while the outline of the diatom is exactly in focus. (It is best to use a low-power eyepiece at this stage.) The illumination is now 'critical' (see Text-fig. 1). Next remove the eyepiece and substitute the bored cork. Put the large stop in front of the lamp, and with one hand hold a sharp pencil with its point in the centre of the stop. Hold the eye near the cork (the purpose of which is to make it easy to keep the line of vision axial) and look down the tube of the microscope. Accommodate the eye for distant vision. (Most microscopists do this reflexly when they look through a microscope. If special glasses are used for distant vision, they should be worn.) *Now focus the condenser slowly upwards until the point of the pencil is clearly seen.* Stop focusing upwards directly this is achieved. If you go too far, focus down again. The proper position has been achieved when a very small movement of the condenser downwards would cause the pencil-point to appear violently distorted and then vanish.

You have now secured what I call 'high-condenser illumination'. The principle of it is illustrated in the central diagram of Text-fig. 1. The condenser having been raised above its so-called 'critical' position, the image of the pencil-point has been brought above the diatom into the front focal plane of the objective, which thus throws parallel rays from it up the tube. The eye, being focused for distant vision, receives these rays and one sees the pencil-point. The objective is now being used as an eyepiece.

Discard the pencil and put the small stop in front of the lamp. On looking through the cork you will see the stop *not filling the whole of the back lens of the objective.* We here deliberately use a stop that will not fill the back lens with light. (To prevent any possibility of misunderstanding I mention here that the iris diaphragm below the condenser must be fully open throughout the whole procedure.)

Keeping the eye at the hole in the cork, grasp the mirror in both hands and turn it in its gimbals in such a way that the circular patch of light (the visual image of the stop in front of the lamp) passes to one side of the back lens of the objective. Stop when about half of the patch of light has passed out of



eyepiece

objective

object

condenser

stop in front
of lamp

"critical"
illumination

high - condenser
illumination

low - condenser
illumination
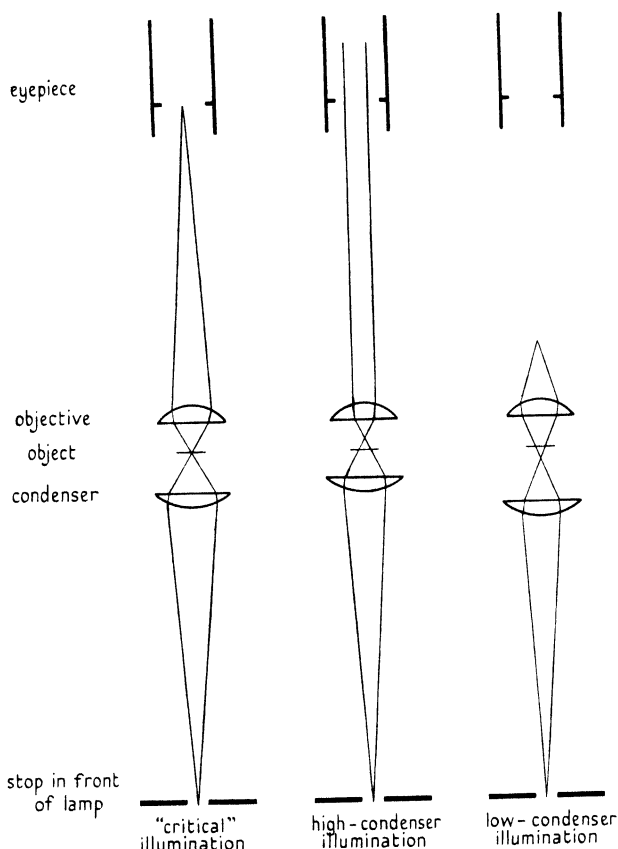
TEXT-FIG. I. Diagram showing the passage of rays of light in the microscope, with the condenser in three different positions.

view and the other half remains at the edge of the back lens. (The circle of light becomes distorted into an oval as it passes towards the edge.) Suppose you have moved the light towards the east: now bring it slowly round the edge of the back lens past the south-east towards the south. As it approaches the south, a spectrum will be seen at the edge of the back lens of the objective a little to the east of north (not to the west of north, as one might expect); this spectrum will move towards the north and will be due north when the direct light is due south. The violet end of the spectrum will be directed

towards the centre of the back lens. Now tilt the mirror to and fro to bring the light a short distance towards the middle of the back lens and back towards the edge of the back lens again, keeping it in the north–south line. Meanwhile watch the spectrum. Leave the mirror in the position that makes the spectrum as bright and complete as possible. The more of the red end of the spectrum you can bring within the back lens, the better. Text-fig. 2 shows the appearance that should be obtained. (If a glass mirror be used, there will be faint additional images of the stop, not quite coinciding with the bright image.)
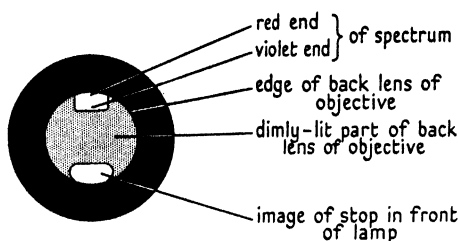


TEXT-FIG. 2. Diagram showing the appearance seen on looking through the hole in the cork, when the direction and degree of obliquity of the light are correct.

Remove the cork and place the high-power eyepiece in position. The striations of the diatom will now be clearly resolved. There is no need whatever to use a colour-screen, even with a non-apochromatic objective. If perfection is desired, focus carefully with the fine adjustment, replace the eyepiece by the cork, and make very small movements of the mirror until the best possible spectrum is obtained; then replace the eyepiece.

In whatever direction the diatom happens to be lying, the same procedure should be adopted (with the obviously necessary changes).

Mr. W. E. Watson-Baker very kindly visited me in Oxford to see a demonstration of the method just described. He allows me to say that the striations of *Amphipleura pellucida* were resolved more quickly than by any other method he has ever seen, and that the images produced were superior to any obtainable by an oblique-light stop placed below the condenser. These comments are mentioned in the hope that the opinion of such an experienced microscopist will encourage others to give the method a trial.

It might be thought that the chromatic and other corrections of the objective would be upset by placing the condenser above its 'critical' position. To investigate this, I have made a careful examination of a number of species of test-diatoms, using central (not oblique) high-condenser illumination. I can find no evidence that the image is either better or worse than with 'critical' illumination. The expression 'critical' illumination is indeed a bad one, for it begs the question whether this is better than other methods. Hartridge (1919) has denied on both theoretical and practical grounds that

'critical' is necessarily preferable to other kinds of illumination: it is simply an easy and reliable way of getting good results.

If the substage iris diaphragm is somewhat closed and then kept in the same position while the two methods of illumination are tried, a larger area of the back lens of the objective will be filled with light by high-condenser than by critical illumination (provided that a sufficiently large stop is used in front of the lamp when the condenser is in the high position). When one is studying diatoms such as *Navicula lyra*, which are not well seen under full-cone illumination, this fact must be kept in mind; for otherwise there will be a danger of using too wide a cone of light when the condenser is in the high position. My attention was called to this point by Dr. O. L. Thomas, who has been kind enough to examine this new method of illumination.

One advantage of high-condenser illumination is that a perfectly structureless source of light need not be used, since the image of the light is not focused in the plane of the object; further, there cannot be any interference with the image by dust on any screen or cooling-chamber that may for any special purpose be placed between the lamp and the condenser.

The direction and degree of obliquity of the light may also be controlled by what I call 'low-condenser illumination', which is explained in the third diagram of Text-fig. 1. The condenser is here placed *below* the 'critical' position, and the source or light is thus focused below the object. An actual image of the source of light is now produced just behind the back focal plane of the objective, where it can be examined, if desired, by screwing a low-power objective into the bottom of the draw-tube. This is not nearly such an effective method for obtaining oblique illumination as that described above, but it is useful in phase-contrast microscopy. If an annular stop be put in front of the source of light, its image will be produced just behind the back focal plane of the objective, where the phase-plate may conveniently be placed.

Dr. R. Barer has kindly read this paper and given me the benefit of his criticism. He remarks that the method of illumination that I adopt is best described by saying that the aperture-stop, usually situated just below the condenser, has been removed to a considerable distance, so that the mirror lies between the aperture-stop and the condenser. I thank Mr. I. C. J. Galbraith, who gave valuable advice on an important theoretical point while I was engaged in perfecting the method of oblique illumination described in this paper.

## SUMMARY

The purpose of the method is to facilitate the resolution of extremely fine regularly-repeated markings on microscopical objects. Resolution is obtained by using the objective as an eyepiece to observe the *direction* and *degree of obliquity* of the light; these are controlled by movements of the mirror. To enable the objective to be used in this way, the condenser is raised above the

position required for so-called 'critical' illumination. The diffraction-spectrum formed by the markings on the object is clearly seen while the mirror movements are being made, and it is easy to produce a bright and complete spectrum.

### REFERENCE

HARTRIDGE, H., 1919. Journ. Quekett micr. Club, **14,** 73.

# Tests by Tissue Culture Methods on the Nature of Immunity to Transplanted Skin

BY

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W HEN skin is grafted from one human being or one rabbit to another, a 'defence' mechanism is called into action which leads, in due course, to the complete destruction of the foreign grafted tissue. A quantitative analysis of the phenomenon (Gibson and Medawar, 1943; Medawar, 1944, 1945, 1946*a*, *b*) has shown that it conforms in broad outline with a reaction of actively acquired immunity. In other words, a human being at first submits to and later recovers from an 'attack' of foreign skin in much the same way as he recovers from an attack of measles: natural (i.e. ready-made) immunity is absent or ineffective; resistance develops in the course of exposure; and recovery is followed by a refractory or 'immune' state. This interpretation is not new, for certain critical tests bearing upon it were made by Peyton Rous in 1910; but it needs emphasizing, because Loeb (1921, 1930, 1945) has for many years denied that immunity in this technical sense has any important part to play in the organism's reaction against tissue grafted to it from other members of its own species.

More specifically, a skin homograft builds up a systemic reaction against itself at a rate which varies with the antigenic relationship between donor and recipient and with the quantity of foreign tissue that is grafted. A second homograft, transplanted from the same donor to the same recipient when the reaction against its predecessor is complete, survives for a few days in a vegetative condition in which cell division is partly or wholly suppressed, and then undergoes accelerated breakdown (Medawar, 1946*a*).

Skin transplantation immunity is thus easily recognizable *in vivo* by the regression of foreign homologous grafts and the refractory state which follows it. If it conformed in detail, as it does in main outline, with the pattern of immunity created by bacterial and other crudely foreign antigens, then one might expect to find some immune body in the tissue or body fluids of an immunized animal which is inimical to the growth *in vitro* of the tissue responsible for generating the immune state. Many years' experience of tumour transplantation has led to the belief that no such factors exist, and it is hard to be convinced by the few tests of the hypothesis that have been said to have had a positive outcome (cf. Phelps, 1937). The purpose of the present paper is to reinvestigate the problem systematically, by taking advantage of newly devised methods that make possible the cultivation *in vitro* of adult skin.

PLAN AND METHODS

The tests to be described in this paper consist in the cultivation of adult rabbit skin epithelium in the presence of serum, tissues, and tissue extracts derived solely from a rabbit heavily and specifically immunized against it. Adult skin epithelium will proliferate and migrate promptly and vigorously when cultivated by flotation upon a stirred and properly aerated serous *fluid* medium (Medawar, 1948*a*). The advantages of fluid culture are its ease of execution, the rapidity with which cell division and migration begin and proceed, and the fact that the cells preserve their normal histological appearance and functional activity during cultivation (Text-figs. 1–5). Its only disadvantage is that, since migratory *over*growth or self-encystment takes the place of outgrowth as it normally occurs *in vitro*, histological analysis must, of necessity, turn upon the use of stained serial sections.

Each single test has made use of an independent pair of rabbits: a donor (D) and a recipient (R). In the initial immunizing operation, eight 'pinch' grafts, each about 10 mm. in diameter and together weighing 0·45–0·55 g., were transplanted from the thigh of D to the chest of R (Medawar, 1944, 1945). (In many trials the recipient was grafted on a second occasion from the same donor.) When the complete and long-standing necrosis of these immunizing grafts gave direct evidence of the completeness of immunization, but in no case earlier than the fifteenth day following their transplantation, a number of very thin 3 mm. × 3 mm. skin squares (minute 'Thiersch' grafts) were cut from a lightly vaselined area on D which had been toughened and rendered slightly hyperplastic by shaving 3 or 4 days beforehand (see Medawar, 1948*a*). The skin squares were thereupon cultivated for 4 or 8 days by flotation, raw side down, on serum freshly withdrawn from the median ear artery of R in dilatation (Medawar, 1946*b*), the serum being used either plain, or as an extractive for R tissues, or, most commonly, as a vehicle for the simultaneous cultivation of a variety of tissues from R. Although the D skin explants usually coalesced with at least some of the R tissue fragments cultivated with them, it was important in some special trials to make quite certain that coalescence and intimate tissue union should take place. In these special trials, therefore, R tissue fragments were glued to the dermal sides of the D skin explants with citrated and recalcified R plasma before cultivation began (see Medawar, 1948*a*).

*Culture methods.* Two culture methods were used: cultivation for 4 days in roller tubes, in a gas phase of air, or for 4–8 days in 'rocker flasks' of 250 ml. capacity in a gas phase of air-oxygen mixture (65–70 per cent. $O_2$). The design and use of the appropriate apparatus has been described in full elsewhere (Medawar, 1948*a*). The rocker-flask cultures were left undisturbed in the incubator for the run of each experiment. With roller-tube cultures the fluid part of the medium was replaced after the second day—with freshly withdrawn serum, if serum alone had been used, but with serum stored for 2 days in the refrigerator if the recipient animal had been killed at the beginning of the

experiment to provide 'immune' tissue explants. The main purpose of using high-$O_2$ rocker-flask cultures was to make certain of the continued functional survival of R spleen- and lymph-node tissue. Neither grows well, if at all, at low oxygen tensions (Parker, 1936, 1937).

Controls were achieved in two ways: either by the growth of D skin in normal homologous serum, tissues, and tissue extracts—i.e. in a medium derived
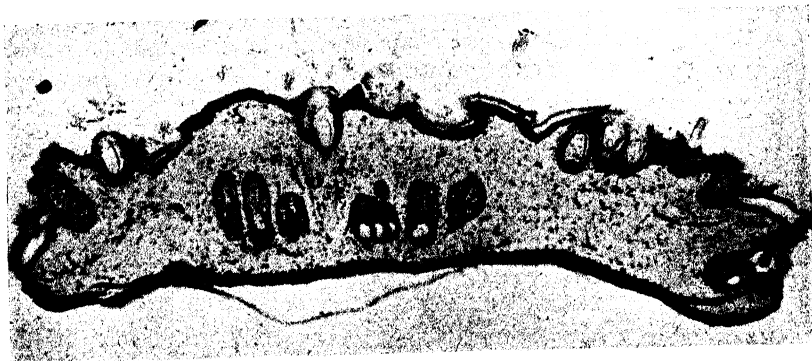


Text-fig. 1. Exp. 18, Table 1. A 4-day roller-tube culture of donor skin in immune serum. Complete self-encystment (****) by migratory overgrowth. Ehrlich's haematoxylin and eosin. ×44.

from a rabbit immunized neither against D cells nor any others; or by cultivating R skin in the same vessel as D skin in media which, being derived solely from R, should in theory be inimical only to the growth of cells from D. (The R skin explants were distinguished from their companions by being cut to a triangular shape.) Controls of these two types are adequate to reveal any non-specific action by the ingredients of the culture medium. It should be noted that a third type of control, the cultivation of skin from a second rabbit in media derived from a recipient specifically immunized against a first, is not acceptable; for although the skin immunity reaction is strongly donor-specific (Medawar, 1946a), the second donor might well happen to share with the first some antigens not also present in R. Some degree of immunity would in that event be directed against it.

In all except the earlier roller-tube experiments (Table 1, exps. 1–16), 9 volumes of the chosen culture medium were mixed before use with 1 volume of streptomycin solution (200 u./ml.) in Ringer. In the rocker-flask tests (Table 2), 8 volumes of the culture medium were mixed with 1 volume of streptomycin solution and 1 volume of 5 per cent. glucose solution in water. With these exceptions, and with the substitution of Krebs-Ringer-bicarbonate for serum in two specific trials (Table 1, exps. 34, 35), the culture medium for D skin was derived wholly from the animal immunized against it.

## METHODS OF ANALYSIS

Three separate routine tests and analyses have been made on roller-tube explants of donor skin in immune media: (1) a test of their continued survival; (2) a measure of the frequency of cell divisions in the epidermis; and (3) a measure of the migratory activity of the epithelium as a whole. Rocker-flask cultures in a high-$O_2$ gas phase were used mainly for the histological analysis of donor skin and immune mesenchymal tissue growing in intimate union.

1. *Survival Test.* So much of importance turns upon whether donor skin survives cultivation in immune media that the diagnosis of survival has not



Text-fig. 2. Exp. 7a, Table 2. A 4-day rocker-flask culture of donor skin in immune serum containing lymph-node fragments, which have not attached themselves to the skin explant (contrast Text-fig. 9). Note the vigorous epidermal proliferation and the relatively subdued migratory activity. Ehrlich's haematoxylin and eosin. ×44.

been allowed to rest on histological evidence alone. Instead, each D explant was duplicated; one was reserved for histological analysis and mitotic counting (see below), and the other tested for its continued survival by the simple process of grafting it back to a large raw area on the animal from which it originally came. Not less than two such tests of survival have been done for each type of medium to which the roller-tube explants were subjected (Table 1, col. 5). The test has been described in full in earlier papers (Medawar, 1947, 1948a).

2. *Mitotic Activity.* The other member of each pair of D skin explants was rinsed in Ringer, fixed in Bouin's solution, and embedded in wax. Alternate sections from strips of 10 taken at 5 evenly spaced vertical levels through the block were stained with Hance's variant of Heidenhain's haematoxylin. All the mitoses in the 5 alternating $8\mu$ sections from the strip of median level were counted and averaged, the figure entered in column 6 of Table 1 representing the mean number of mitoses per median $8\mu$ section divided by the width of the explant in millimetres.

3. *Migratory Activity.* A median section stained with Ehrlich's haematoxylin was marked for degree of epidermal migration in accordance with the scale which follows (Table 1, col. 7):

* Overgrowth incipient: the 'shoulders' of the explant rounded off.
** Overgrowth about 1/3rd complete.
*** Overgrowth about 2/3rds complete.
**** Complete self-encystment (cf. Text-figs. 1, 3, 4, 5).

## RESULTS: ROLLER-TUBE CULTURES

The results of 37 tests are summarized by Table 1 and amplified by the notes given below, which correspond to the references in column 4. The use



TEXT-FIG. 3. Tissue coalescence test. A 4-day roller-tube culture of donor skin to the under side of which small fragments of kidney medulla have been glued with immune plasma. Culture medium: immune serum. Epidermal and medullary epithelium between them have brought about complete self-encystment of the explant. Ehrlich's haematoxylin and eosin. × 40.

of streptomycin at a final concentration of 20 u./ml. in the culture medium was adopted from exp. 17 onwards. The use of minute 'Thiersch' grafts as explants (mean diameter after fixation: 2·9±0·1 mm.) was not adopted until exp. 14; small 'pinch' grafts, much thicker at the centre and somewhat broader (4·0±0·2 mm.) had been used until then, and this probably accounts for the fact that none of the explants quite achieved complete self-encystment (****) in 4 days. The total weight of the immune tissue explants added to the culture medium in various experiments did not exceed 4 mg.; each was cut into a cube not exceeding, and so far as possible not much less than, 1½ mm. in length of side. In all the experiments described in this paper, the lymph-node explants were taken from the axillary node receiving the lymphatics from the side of the chest carrying the immunizing homografts.

TABLE I. *Roller-tube cultures: the results of 37 tests of the growth of donor skin explants in media of various types derived from rabbits specifically immunized against them.*

The explants have been marked for survival by the transplantation test (see text), and for mitotic and migratory activity. The entries in column 4 refer to the Notes in the text.

| No. | Donor→ Recipient | Culture medium | Notes | Survival | Mitoses | Migration |
|---|---|---|---|---|---|---|
| 1 | 396→397 | Immune serum . . . . | .. | * | .. | *** |
| 2 | 401→402 | Immune serum . . . . | .. | * | 0·6 | ** |
| {3 | 411 | Normal homologous serum (from 413) | .. | .. | 1·2 | ** |
| {4 | 411→412 | Immune serum . . . . | .. | * | 1·2 | * |
| {5 | 416 | Normal homologous serum (from 413) | .. | .. | 1·0 | *** |
| {6 | 416→417 | Immune serum . . . . | .. | * | 0·6 | *** |
| {7 | 421 | Normal homologous serum (from 416) | .. | .. | 0·7 | ** |
| {8 | 421→422 | Immune serum . . . . | .. | * | 0·7 | ** |
| 9, 10 | 426→427 | {Immune serum . . . . <br> {Immune serum + lymph-node explants . . . . | .. <br> .. | .. <br> * | 0·9 <br> 1·5 | * <br> ** |
| 11, 12 | 431→432 | {Immune serum . . . . <br> {Immune serum + lymph-node explants . . . . | .. <br> .. | .. <br> * | 1·1 <br> .. | ** <br> .. |
| 13 | 436→437 | Immune serum + spleen explants . | .. | * | .. | .. |
| 14 | 442→443 | Immune serum + spleen explants . | .. | * | 1·8 | ** |
| 15 | 445→447 | Immune serum + liver explants . | .. | * | 0·6 | * |
| 16 | 451→453 | Immune serum + liver explants . | .. | * | 0·4 | ** |
| 17 | 459→460 | Immune serum + leucocytes . . | a | * | 1·1 | *** |
| 18 | 461→462 | Immune serum (adjuvant immunization with chicken plasma) . . | b. Text-fig. 1 | * | 0·9 | **** |
| 19 | 469→470 | As exp. 18 . . . . | b | .. | 0·7 | **** |
| 20 | 471→472 | Immune serum + leucocytes . . | a | * | 1·2 | ** |
| 21 | 473→474 | Immune serum as extractive for liver and spleen tissue; adjuvant immunization with chicken plasma . | bc | * | 0·9 | *** |
| 22, 23 | 475→476 | {Immune serum + spleen explants . <br> {Immune serum + lymph-node explants . . . . | Text-fig. 4 | .. <br> .. | 1·4 <br> 1·3 | **** <br> **** |
| 24 | 478→479 | Immune serum as extractive for liver and spleen tissue, with liver and spleen explants; adjuvant immunization with chicken plasma . . | bc | * | 0·7 | *** |
| 25 | 481⇌482 | R skin in D serum + liver and spleen explants; 'reverse' test . . | d | * | 0·8 | **** |
| 26 | 488⇌489 | As exp. 25 . . . . | d | * | 1·1 | **** |
| 27 | 519→520 | Immune serum + kidney medulla explants . . . . | .. | * | 1·0 | *** |
| 28 | 527→528 | As exp. 27 . . . . | .. | * | 0·7 | *** |
| 29 | 532→533 | 20 per cent. defibrinated whole blood in serum + blood-clot explants . | e | * | 1·2 | **** |
| 30 | 537→538 | As exp. 29 . . . . | e | * | 1·0 | **** |
| 31 | 539→540 | As exp. 29 . . . . | e | * | 2·2 | **** |
| 32 | 545→546 | Immune serum anaerobically . . | f | * | 0 | 0 |
| 33, 34 | 550→551 | {Immune serum anaerobically . . <br> {Ringer-bicarbonate as extractive for kidney and liver tissue . . | f <br> c | * <br> * | 0 <br> 2·1 | 0 <br> **** |
| 35 | 560→561 | Ringer-bicarbonate as extractive for kidney and spleen tissue . . | c | * | 3·9 | **** |
| 36 | 604→605 | 100 per cent. defibrinated whole blood; adjuvant immunization with heterologous cells . . . | eg | * | 1·0 | **** |
| 37 | 608→609 | As exp. 36 . . . . | eg | * | 1·1 | **** |

## Notes on Table 1

(a) *The use of leucocytes* (exps. 17, 20). Leucocytes were present in the immune serum at 2–2½ times their physiological concentration, having been separated from 6 to 8 ml. citrated whole blood by repeated slow spinnings and then resuspended in 3 ml. serum. The serum preserved a strong cellular opalescence during cultivation, though a majority of the leucocytes 'agglutinated' and formed compact cell clusters.

(b) *Adjuvant immunization with chicken plasma* (exps. 18, 19, 21, 24). The recipients were injected with 2 ml. chicken plasma 12 days before and 4 days after the transplantation of the immunizing dose of homografts, the first injection being intradermal and the second intravenous. The antigen dilution titre of precipitins in the R serum exceeded 1,000 when the culture tests were begun 12 days after the second injection.

(c) *Tissue extractions* (exps. 21, 24, 34, 35). About 1 g. of each type of tissue was chopped into a fine mush with scissors and extracted with 10 ml. serum (exps. 21, 24) or 10 ml. Krebs-Ringer-bicarbonate (exps. 34, 35). In each case 2·7 ml. of the supernatant fluid, clarified by spinning, served as the culture medium.

(d) *'Reverse' tests* (exps. 25, 26). The skin of the immunized *recipient* was cultivated in serum and tissue fragments derived from its donor.

(e) *Defibrinated blood* (exps. 29, 30, 31, 36, 37). It proved best to dilute the defibrinated whole blood with 4–5 volumes of its own serum.

(f) *Anaerobic cultivation* (exps. 32, 33). These experiments, though entered here for ease of reference, made use of the anaerobic culture apparatus described by Medawar (1947), and not of roller tubes. In this apparatus the explants were incubated for 4 days at 38° C. in 5 ml. serum under an atmosphere of $H_2$ from which the last traces of $O_2$ had been removed by catalysis with heated palladium (indicator: methylene blue). Skin epithelium incubated anaerobically neither moves nor proliferates in any degree; hence the zero entries in the last two columns. The survival test shows, however, that in immune serum as in normal serum (Medawar, 1947), the skin cells remain alive and resume normal growth and activity when oxygen is restored to them.

(g) *Adjuvant immunization with a cellular antigen* (exps. 36, 37). A fine suspension in Ringer of about 100 mg. adult mouse spleen, lymph node, and submaxillary gland fragments was injected intradermally and intraperitoneally into R on the day of its receiving the immunizing grafts from its homologous donor. In exp. 37 the heterologous cell suspension was, in addition, injected intradermally into the graft donor area of D immediately before the immunizing grafts were cut from it for transplantation to R.

### Tissue Coalescence Tests

Special interest attaches to an additional and independent series of roller-tube cultures in which fragments of 'immune' tissue were glued on to the dermal surfaces of the skin explants before cultivation began (see above). Text-figs. 4 and 5 illustrate the complete encapsulation of lymph-node fragments by skin epithelium that has migrated from the donor explants. In Text-fig. 5 the very pronounced thickening of the epibolic skin epithelium in the immediate neighbourhood of the encapsulated explant shows that, so far from its having had any inhibitory action, the node tissue seems actually to have stimulated the growth of the adjacent epithelial cells. Text-fig. 3 in low power and Text-fig. 8 in higher power illustrate complete confluence between donor skin epithelium and epithelium from the immunized rabbit's kidney medulla. It is hard to tell where the one ends and the other begins.

The tests using lymph-node explants (and others using spleen) are not decisive, because cultivation in a gas phase of air is not adequate to maintain the complete functional survival of tissues of these two types. The experiments to be described in the next section were done mainly to repair this shortcoming.

## RESULTS: ROCKER-FLASK CULTURES

The cultures to be described here were done in 250 ml. gas perfusion rocker flasks containing the following culture ingredients: (*a*) 4–15 ml. immune serum containing streptomycin and glucose at final concentrations of 20 u./ml. and 0·5 per cent. respectively; (*b*) two square or rectangular donor skin explants of the same size as those used in roller tubes; (*c*) two skin explants from the recipient, cut to triangular shape for ease of identification and serving as controls against the possibility of any non-specific action by the ingredients of the medium; and (*d*) up to 88 mg. immune spleen or lymph-node tissue
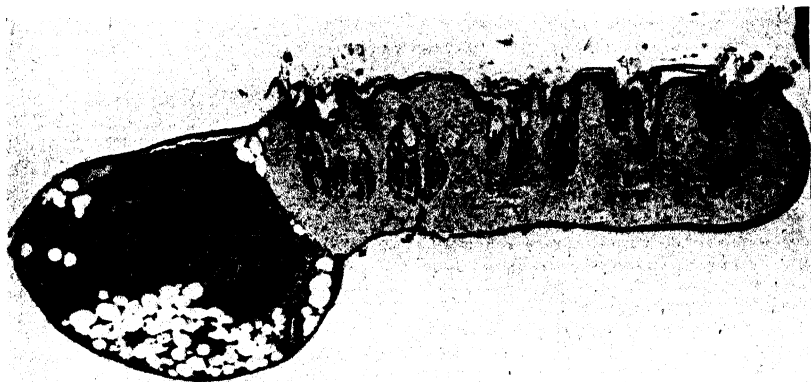


TEXT-FIG. 4. Exp. 23, Table 1. A 4-day roller-tube culture in immune serum containing lymph-node fragments, one of which has by chance attached itself to the edge of the skin explant and become encysted by epidermal epithelium. Ehrlich's haematoxylin and eosin. × 37.

(mainly the former) cleanly chopped into cubical fragments of the size habitually used in tissue cultivation.

The flasks were perfused with one measured volume of filtered cylinder $O_2$ at the timed rate of 100 ml. per minute, giving a final concentration of 65–70 per cent. $O_2$, and then set to rock for from 4 to 8 days without further attention in a dry-air incubator at 38° C. At the end of the experiment the pH of the culture medium was tested with the glass electrode and the cultivated tissues were fixed in formal-$HgCl_2$ for histological analysis. Independent survival tests were done in only two cases, with positive results. Both spleen and lymph-node fragments showed obvious proliferation under these conditions of culture (Text-figs. 6, 7), but the results from the cultivation of spleen were characteristically variable (cf. Parker, 1936, 1937). In most cultures the medium became strongly opalescent with mononuclear cells that had migrated from the spleen explants during the first day or two of cultivation. The occurrence or failure of this response was in no way correlated with the degree of survival of the spleen explants as judged by their histological appearance

at the end of the experiment. Cells migrating from the lymph-node explants showed a strong tendency to infiltrate the dermis of donor and control skin explants alike and to bring about some degree of collagen dissolution.

The behaviour of the skin explants was also variable: there were occasionally greater differences among the members of the two pairs than between them. For this reason many more cultures (Table 2) had to be done than would otherwise have been necessary. In high-$O_2$ media the migratory activity of the skin epithelium was far less pronounced than with cultivation in a gas phase of air, and complete self-encystment was achieved more slowly and less often. Cell division, on the other hand, was rapid and led to the formation of a deeply stratified epidermis (Text-fig. 2).

TABLE 2. *Rocker-flask cultures in a high-$O_2$ gas phase*

Summarizing the various culture-medium volumes, culture periods, and tissue dosages used in control cultures in autologous media (group A) and in experimental cultures in immune media (group B).

| No. | Donor→ Recipient | Volume of medium, ml. | Days of cultivation | Tissue | Final pH | Notes |
|---|---|---|---|---|---|---|
| A. 1 | 669 | 10 | 4 | 72 mg. spleen | 7·81 | 0·2 per cent. NaHCO₃ in culture medium. |
| 2 | 672 | 10 | 4 | 46 mg. spleen | 7·45 | |
| 3 | 679 | 10 | 4 | 36 mg. spleen | 7·65 | |
| 4 | 680 | 6 | 4 | 55 mg. spleen | 7·05 | |
| 5 | 685 | 4 | 4 | 33 mg. spleen | 7·60 | |
| 6 | 685 | 6 | 4 | 37 mg. spleen | 7·60 | |
| B. 7a | 651→652 | 10 | 4 | 50 mg. node | .. | Text-figs. 2, 9. |
| 7b | 651 | 10 | 4 | 50 mg. node | .. | Normal *homologous* serum from 647; no glucose. |
| 8 | 653→654 | 12 | 4 | 30 mg. spleen | .. | No glucose. |
| 9 | 657→658 | 10 | 6 | 25 mg. spleen | 7·65 | Glucose 1 per cent. |
| 10 | 659→660 | 11 | 5 | 80 mg. spleen | .. | Recipient not fully immune. |
| 11 | 663→664 | 10 | 4 | 36 mg. spleen | 7·25 | |
| 12 | 665→666 | 10 | 4 | 38 mg. spleen | 7·05 | |
| 13 | 667→668 | 8 | 4 | 51 mg. spleen | 7·30 | |
| 14 | 686→687 | 10 | 8 | 61 mg. spleen | 6·65 | Medium infected. |
| 15 | 690→691 | 15 | 8 | 47 mg. spleen | 7·20 | |
| 16 | 694→695 | 15 | 8 | 88 mg. spleen | 6·70 | Medium infected. |
| 17 | 696→697 | 10 | 8 | 68 mg. spleen | 6·78 | |
| 18 | 700→701 | 15 | 8 | 36 mg. spleen | 7·25 | |
| 19 | 706→707 | 15 | 8 | Serum alone | 7·15 | |
| 20 | 710→711 | 15 | 8 | 74 mg. node | 7·42 | |
| 21 | 712→713 | 15 | 8 | 55 mg. spleen | 7·45 | |
| 22 | 724→725 | 6 | 4 | 36 mg. spleen | 7·21 | |
| 23 | 726→727 | 8 | 4 | 45 mg. spleen | 7·19 | |
| 24 | 728→729 | 6 | 4 | 49 mg. node | 7·46 | |

*Results.* The cultures which were analysed are set out in summary in Table 2. There was no appreciable difference between the experimental cultures and the controls run in media of autologous origin.

In the experimental group the donor skin explants were quite clearly superior to their controls (i.e. the skin explants taken from the immunized recipient itself) in exps. 15 and 18, and clearly inferior in exps. 12, 14, 22, and

23. (In exp. 14, as a result of infection, the growth of both skin pairs was very poor.) There was no ground for supposing that these differences were anything but fortuitous in origin.

There was almost invariably some degree of union between the skin explants and the fragments of spleen or lymph-node tissue cultivated with them. Text-fig. 9 illustrates a particularly intimate union between strongly proliferating donor skin epithelium and an immune lymph-node fragment almost wholly encapsulated by it. Mitotic figures, everywhere abundant,



TEXT-FIG. 5. Tissue coalescence test. As Text-fig. 4, save that in this case a fragment of lymph node was deliberately glued on to the dermal surface of the skin explant, where it has now undergone complete epidermal encystment. The node tissue is now necrotic, but the epithelium in its neighbourhood is thicker than it is elsewhere. Ehrlich's haematoxylin and eosin. ×44.

were not less frequent in the epibolic epithelium abutting immediately against the node tissue. In short, the entire series of experiments affirms that the mitotic and migratory activity of skin epithelium was in no way influenced by cultivation in media, and in intimate association with tissues, derived from a rabbit heavily immunized against it.

### OTHER SEROLOGICAL TESTS

It has already been established (Medawar, 1946*b*) that the grafting of skin from one rabbit to another does not elicit the formation of red-cell iso-agglutinins. It might conceivably elicit the formation of agglutinins for cell suspensions of donor skin epithelium itself; and to complete the present series of *in vitro* tests the possibility was examined as follows.

About 1 cm.² of the thinnest possible shavings from the vaselined hyper-plastic skin of a donor rabbit was incubated for one hour in a Seitz-filtered 0·5 per cent. solution of commercial trypsin powder in Ringer-bicarbonate at

pH 7·6 (Medawar, 1941). The shavings were thoroughly rinsed in Ringer, and from each one the dermal layer was lifted off with fine forceps, leaving behind a pure epidermal sheet which transplantation tests have shown to be



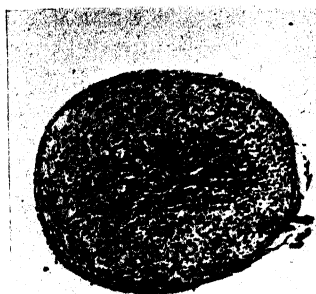FIG. 6                                              FIG. 7
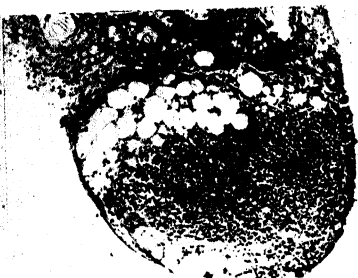


FIG. 8                                              FIG. 9

TEXT-FIG. 6. Illustrating the characteristic appearance of a 4-day spleen fragment cultivated in the high-$O_2$ gas phase of a rocker flask. Ehrlich's haematoxylin. ×70.

TEXT-FIG. 7. As Text-fig. 6: a lymph-node fragment.

TEXT-FIG. 8. See Text-fig. 3: an illustration in higher power of the coalescence of donor skin epithelium with 'immune' kidney medulla epithelium to form a continuous sheet. Ehrlich's haematoxylin and eosin. ×53.

TEXT-FIG. 9. Exp. 7a, Table 2. Portion of a 4-day rocker-flask culture in immune serum containing lymph-node fragments, one of which has attached itself to the margin of the culture and undergone complete epidermal encystment. Note the strongly proliferating epithelium in its neighbourhood, and the complete failure of the 'immune' node tissue to affect its growth adversely. Heidenhain's haematoxylin. ×53.

still living (Billingham and Medawar, 1948). The sheets were flattened down on their vaselined cuticular surfaces and then smoothly and firmly scraped to separate the cells of the deeper layers from those of the cuticle. The clumps so formed were rendered into a fine suspension in Ringer by sucking them

repeatedly in and out of a fine-bore pipette. The suspensions were then mixed in agglutination tubes with $\frac{1}{2}$ or 1 volume of serum from a rabbit specifically and effectively immunized against the donor skin. Five such tests, run for 3–6 hours at body temperature and overnight at room temperature, were uniformly negative in outcome, and the cell suspension which in due course settled in the bottom of the agglutination tubes could be evenly redispersed by light tapping or shaking.

The presence of precipitins for skin extracts was tested simultaneously by the ring method, a water-clear extract being secured by freezing thin skin slices with $CO_2$ snow, cutting them into $15\mu$ sections on a freezing microtome, grinding the sections so cut in a mortar under Ringer, and then spinning down the collagenous and cuticular debris. No ring of precipitation developed between immune serum and the supernatant skin extract, and the subsequent mixing of the two solutions with further incubation produced no turbidity perceptible by direct or oblique illumination.

## Discussion

The experimental results make it clear that immunity to foreign homologous skin grafts is not demonstrable by an *in vitro* reaction of any type described in this paper. Notwithstanding statements that have from time to time been made to the contrary, they justify the provisional conclusion that the occurrence of free serum antibodies is not a *sufficient* explanation of the destruction of foreign homologous tissue. The tests involved the cultivation of not more than 5 mg. donor skin for up to 8 days in so much as 15 ml. of serum and 88 mg. of mesenchymal tissue derived solely from a rabbit specifically and demonstrably immunized against it. Within these limits the experimental results are quite decisive: skin epithelium survives, moves, and proliferates with unaffected vigour in these presumptively immune media.

The results are consistent with those of Harris (1943), which have not yet been fully reported on; and, also, with what is known of the relationship between the regression of tumour homografts and the occurrence in their hosts of antibodies revealed by complement fixation. Actively induced complement fixing iso-antibodies appear in the sera of rabbits bearing a variety of transplanted tumours (Friedewald and Kidd, 1945) and they react *in vitro* with the fine particulate matter thrown down from saline tissue extracts by spinning at about 20,000 $g$ for 2–3 hours. An antibody elicited by at least one such tumour, the Brown-Pearce carcinoma, has since been shown to be specific to the tumour in question (MacKenzie and Kidd, 1945; Kidd, 1946). The lack of correlation between tumour regression and specific antibody titre makes it clear, however, that 'factors other than the specific antibody are probably responsible for regression of growth in the majority of cases' (Kidd, 1946).

Two recent attempts have been made to elicit the formation of iso-antibodies by injecting extracts or homogenates of foreign homologous tissues together with toxins of known antigenic power (Schwentker and Comploier,

1939; Hecht, Sulzberger, and Weil, 1943). In neither case was the homologous tissue by itself effective.

Four possibilities that indicate the limitations of the present preliminary analysis may now be briefly discussed. First, it is possible that cultivated tissues will survive for long periods, but not indefinitely, in media derived from animals immunized against them. What is known already about the tempo and vigour of the immune reaction *in vivo* gives one no ground for supposing this to be true. A second possibility is that the dosage relationships between the cultivated tissues and their media were inappropriate to the demonstration of an immune reaction. When a skin graft weighing about 0·05 g. is transplanted to a 2 kg. rabbit, it 'competes' with 40,000 times its own mass of native tissue. The corresponding dosage ratio for the tests *in vitro* reported here has not exceeded 20 at the outside. This second possibility will therefore be investigated further, though it depends upon an interpretation of the immune reaction—in terms of a theory of 'competition'—which is supported only by perhaps remote analogies with the behaviour of microorganisms.

A third possibility, which preliminary tests suggest to be the one most worthy of further inquiry, is that immune media affect skin epithelium *in vitro* only in such a way as to expedite its breakdown on subsequent grafting. Woglom's (1933, 1937) experiments with transplanted rat sarcomata support this view, and Kidd (1946) has found that a preliminary incubation of Brown-Pearce tumour tissue in media containing its specific antibody reduced or suppressed its growth on transplantation. Finally, it may be that the hypothetical antibodies are cell-bound, and that an accessory immune mechanism is needed to bring them into action, viz. the destruction of the host's antibody containing cells in the immediate neighbourhood of the graft. (The regression of skin grafts is accompanied by the wholesale destruction of such native lymphocytes and other leucocytes as may have penetrated them: cf. Medawar, 1944.)

The biological significance of the tissue homograft reaction deserves some mention. It is widely believed that tissue transplantation, like blood transfusion, is an act that has no counterpart in nature. This is clearly untrue: the mammalian foetus is a tissue homograft, though it is normally protected from the consequences of that fact by its strictly independent circulation. (The behaviour of skin homografts in the anterior chamber of the eye is to some extent a model of the mother-foetus relationship: such grafts, if not vascularized, are not destroyed: cf. Medawar, 1948*b*.) It is at present thought likely (cf. Penrose, 1946; Kalmus, 1947) that a variety of foetal and placental abnormalities are to be attributed to immunological incompatibilities between foetus and mother rather than, for example, to endocrine disorder. If this view is correct, the interpretation of some forms of foetal abnormality is likely to turn on the analysis of the tissue homograft reaction.

Text-figs. 1–5 are from photographs taken by Mr. D. A. Kempson. The

## SUMMARY

The transplantation of skin from one rabbit to another elicits a reaction that conforms in main outline with that of an actively acquired immunity. The experiments described in this paper were designed to test the hypothesis that the regression of such grafts is secured by the action of antibodies demonstrable *in vitro*.

Skin from adult rabbits has therefore been cultivated in the presence of serum and growing mesenchymal tissues derived solely from rabbits heavily and specifically immunized against it.

Immune sera and tissues are without effect on the survival, cell-division frequency and migratory activities of explanted skin, and agglutinins for epidermal cell suspensions are not demonstrable in immune sera.

With certain stated qualifications, it has therefore been concluded that the occurrence of free antibodies is not a sufficient explanation of the regression of skin homografts *in vivo*.

## REFERENCES

BILLINGHAM, R. E., and MEDAWAR, P. B., 1948. Heredity, **2**, 29.
FRIEDEWALD, W. F., and KIDD, J. G., 1945. J. exp. Med., **82**, 21.
GIBSON, T., and MEDAWAR, P. B., 1943. J. Anat. Lond., **77**, 299.
HARRIS, M., 1943. J. exp. Zool., **93**, 131.
HECHT, R., SULZBERGER, M. B., and WEIL, H., 1943. J. exp. Med., **78**, 59.
KALMUS, H., 1947. Ann. Eugen., **13**, 146.
KIDD, J. G., 1946. J. exp. Med., **83**, 227.
LOEB, L., 1921. Biol. Bull., **40**, 143.
—— 1930. Physiol. Rev., **10**, 547.
—— 1945. *The Biological Basis of Individuality*. Springfield.
MACKENZIE, I., and KIDD, J. G., 1945. J. exp. Med., **82**, 41.
MEDAWAR, P. B., 1941. Nature, **148**, 783.
—— 1944. J. Anat. Lond., **78**, 176.
—— 1945. Ibid., **79**, 157.
—— 1946a. Brit. J. exp. Path., **27**, 1.
—— 1946b. Ibid., **27**, 15.
—— 1947. Quart. J. micr. Sci., **88**, 27.
—— 1948a. Ibid., **89**, 187.
—— 1948b. Brit. J. exp. Path., **29**, 58.
PARKER, R. C., 1936. Science, **83**, 579.
—— 1937. Ibid., **85**, 292.
PENROSE, L. S., 1946. Ann. Eugen., **13**, 141.
PHELPS, H. J., 1937. Amer. J. Can., **41**, 441.
ROUS, P., 1910. J. exp. Med., **12**, 344.
SCHWENTKER, F. F., and COMPLOIER, F. C., 1939. J. exp. Med., **70**, 223.
WOGLOM, W. H., 1933. Amer. J. Can., **17**, 873.
—— 1937. Ibid., **30**, 477.

# Budding and the Reproductive Cycle of *Distaplia*

BY

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THE process of budding and the production of tadpole larvae in *Distaplia* have long been of interest and have been the subject of many contributions. At the same time, while a great deal is now known about certain phases of the developmental cycle, other parts have remained irritatingly obscure, so much so that Julin (1896), for example, concluded that only tadpoles produced buds and that colonies were formed from tadpoles, with buds, that had failed to escape from the colonial mass. Studies to date have been made on one species, *Distaplia magnilarva*, and in relation to the nature of the tadpole and its metamorphosis, its production of buds, and the development, as distinct from origin, of buds in the adult colony. The present account is an attempt to describe the whole developmental cycle, based mainly on abundant material of *D. bermudensis* collected from the Gulf coast of Florida, with comparative material of *D. rosea, D. garstangi, D. clavata*, and *D. magnilarva*.

The most recent study, known only after the present investigations were completed, is that of Brien (1939) and is based upon *D. magnilarva*. It concerns solely the process of budding in the larvae, and the general conclusion is similar to that of Julin that the buds produced by the tadpole larvae give rise directly by continued budding to all the zooids comprising a colony. This appears to be a misinterpretation of the process occurring in the larva and the result of failure to discover any other source of buds from later stages. Such failure is by no means the result of lack of effort, and there is no doubt that both Julin and Brien made an intense and competent search for an origin of buds from adult zooids. The reason lies more with the nature of the species examined, and without doubt, had the present investigation been confined to a study of *D. magnilarva* and *D. rosea*, the problem would have remained unsolved. This question will be discussed further in the last section of this paper.

The present account is divided into three parts, representing three phases of the life-cycle. Any one of the three might have been the commencement since we are dealing with a cycle without a real beginning or an end. As a somewhat arbitrary starting-point the attached and newly active oozooid has been taken, with the first section consisting of the growth of zooids to sexual maturity involving successive asexual generations, together with the general development of buds up to and beyond the active functional state. The second

section concerns the development and nature of the tadpole larva, while the third concerns the regression of old zooids, the origin of buds from such zooids, and the first stages of morphogenesis in the bud. A comparative discussion is left to the end.

### PART I. ORIGIN, NATURE, AND DEVELOPMENT OF ZOOIDS

This part is divided into three subsections, one concerning observations on *Distaplia rosea*, one on *D. bermudensis*, and a comparative section.
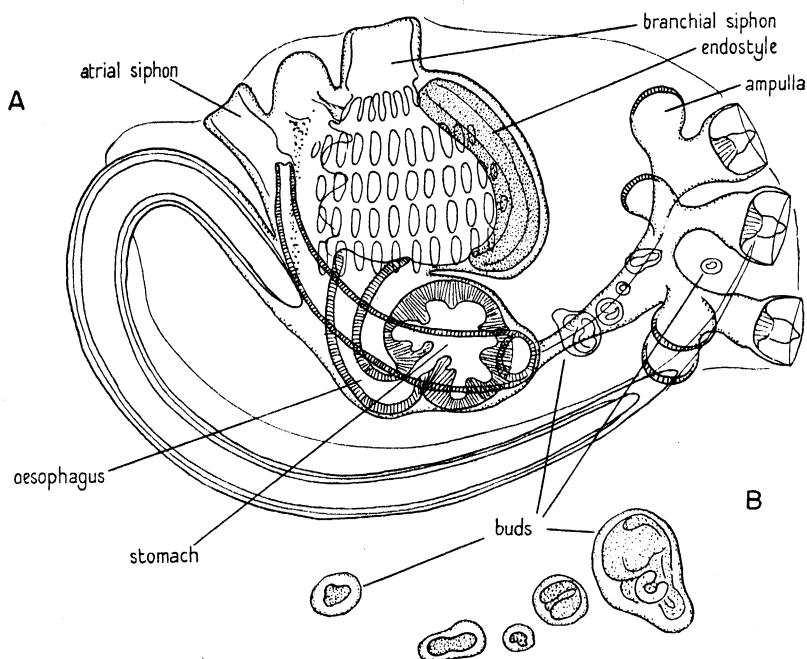


TEXT-FIG. I. A. Tadpole of *D. rosea*, showing almost equivalent development of permanent and transient structures and series of buds. B. Bud series, enlarged and in reversed order.

### *Distaplia rosea*

As an introduction, the tadpole larva is shown (Text-fig. 1A) not to emphasize the tadpole structure as such but the more permanent structures. These are more readily defined in a negative sense and comprise the larva with the exception of the tail, sensory vesicle, and the three anterior adhesive organs. This leaves the ascidiozooid with its siphons, endostyle, branchial sac, intestinal loop and heart, together with four epidermal ampullae and a number of buds enclosed within the larval test. In the example shown five buds of varying size are present.

After settling, the tadpole structures are resorbed and the oozooid becomes functional as such, and is shown in Text-fig. 2A. The four ampullae survive as the permanent organs of attachment after the disappearance of the temporary adhesive organs. During the active life of the oozooid the buds do not grow to any appreciable extent. After a certain period, to be measured in days in the case of *D. rosea*, weeks in the case of *D. bermudensis*, the oozooid undergoes resorption and finally disintegrates within its test. Coinciding with this
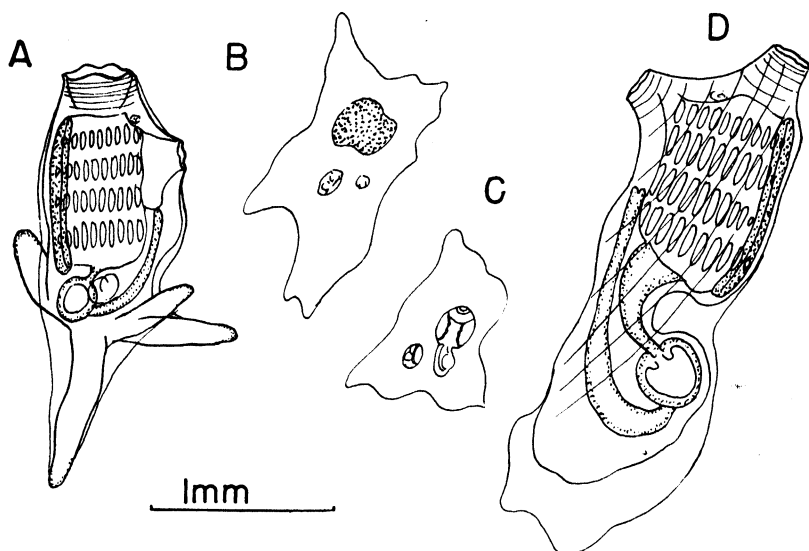


Text-fig. 2. A. Fully metamorphosed oozooid of *D. rosea*. B. Resorbed oozooid and two surviving buds. C. Two buds of first blastozooid generation, one developing and one abortive. D. Fully developed first blastozooid.

resorption, the largest of the buds grows rapidly and the first blastozooid replaces the oozooid. In none of the many cases observed did the second generation ever consist of more than the one active blastozooid. Usually a second bud, apparently arrested at an early stage of development, can be seen, but with no sign of the remaining and smaller buds originally contained in the larval test. The following table expresses the duration of the various phases at 20° C. kept in finger-bowls submerged in running seawater.

*Duration of phases of cycle at 20° C.*

| | |
|---|---|
| Active free-swimming period of tadpole . . . . | 2 hours |
| Period from tadpole stage to functional oozooid . . . | 5 days |
| Survival of functional oozooid . . . . . . | 16–24 days |
| Period of resorption or degeneration of oozooid . . | 12–24 hours |
| Subsequent developmental period of first blastozooid . . | 10–15 days |
| Survival of first blastozooid . . . . . . | 8–10 days |

The first blastozooid differs little from the established oozooid. It is somewhat larger, and while the gill slits remain unchanged as four rows of definitive stigmata as in the oozooid, the gut loop is considerably longer and better displays the divisions and appearances of that of mature zooids. Neither oozooid nor first blastozooid shows any observable sign of gonads. The survival periods for the oozooid and blastozooid, as indicated above, may be
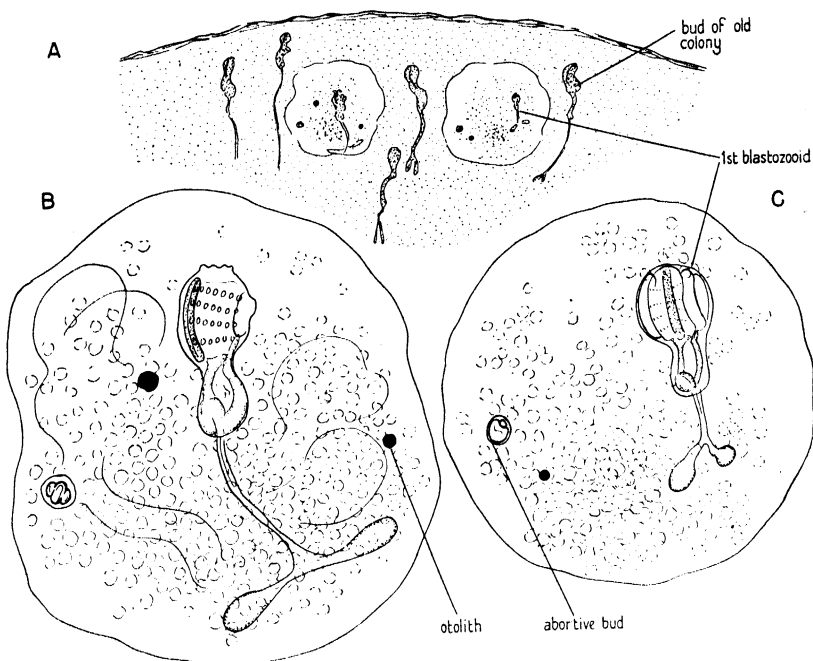


TEXT-FIG. 3. A. Part of upper surface of a corm of *Distaplia rosea* after zooids resorbed and surface smooth, containing developing blastozooids and two incorporated young colonies resulting from retained tadpoles. B, C. The same two second-generation colonies enlarged to show in each case a developing first blastozooid, an abortive blastozooid, and remnants of the sense organs and ampullae of the oozooid.

abnormally brief, for aquarium conditions at their best may be none too good, and it is possible that at lower temperatures, with more abundant food organisms, and the purer water of the natural environment, both forms might survive for a much longer period. What does appear to be established, however, is the comparative instability of the active zooid and that it does have a limited existence, one generation of zooids replacing another and being above all dependent for their own growth and development upon the degeneration products of the resorbing generation.

Julin (1896), working mainly with *D. magnilarva* and failing to find the source of new buds in old colonies, suggested that buds were replenished

from tadpoles produced within the colony by the mature zooids. He brought forward no positive evidence for this and Daumezon (1909) rejected the hypothesis on *a priori* grounds alone. However, in one of several small but fully mature colonies of *D. rosea* that have been sectioned, a situation conforming exactly to Julin's concept undoubtedly existed, for side by side with easily recognized young developing buds a number of metamorphosed larvae were unmistakably present. The limits of each larval unit were sharply shown by the clearness and firmness of the test substance of the young colonies compared with that of the parent colony in which they were embedded (Text-fig. 3). Further proof that they were what they seemed to be was furnished by the fact that all stages were found from intact but trapped tadpole larvae to forms in which not only was metamorphosis completed, but the oozooid itself resorbed. In the last and most advanced cases, the oozooid was represented by surviving traces of its original ampullae together with the persisting pigmented otolith and ocellus (Text-fig. 3B). The first blastozooid was present in each, together with one other abortive bud of the same generation, while no others were discernible. Therefore, tadpoles may inadvertently become retained in the parent colony, and if so, may undergo metamorphosis and give rise to a first blastozooid generation upon dissolution of the oozooid. It is to be noted that only one of the original four or five buds actually develops, presumably the largest, and that the others fail to develop and even disappear under conditions clearly optimal for their development.

### *Distaplia bermudensis*

The foregoing account of *D. rosea* is to some extent introductory, but has been emphasized since it comprises the bulk of the observations that have been made upon living material. Colonies of *D. rosea*, however, are tiny, rather difficult to obtain, and hardly afford an adequate material basis for a general study. Colonies of *D. bermudensis* may also be rather small, as, for example, most colonies found in the Bermudas proper, but along the shores of the Gulf of Mexico colonies of a *Distaplia* that appear to be the same species may attain relatively enormous sizes, and it is this type, collected at Englewood, Florida, that has been used in the following account.

### *Structure of zooid*

A large active zooid is shown in Text-fig. 4A, drawn to the same scale as the oozooid and first blastozooid of *D. rosea* in Text-fig. 2. As in these, the branchial sac has four rows of dorso-ventrally elongated stigmata on each side. The gut loop is differentiated into an oesophagus, stomach, post-stomach or mid-intestine, and a long rectal ascending limb opening anteriorly into the atrial cavity. The gonads lie in the loop of the intestine, and both sperm duct and oviduct follow the ascending limb of the intestine to open beside the anus. Extending into the depths of the common test of the colony from the posterior end of the zooid are a pair of closely applied vascular tubes. They communicate with one another usually in two regions, often near their distal ends,

and usually also a relatively short distance from the body of the zooid at a junction marked by ampullary swellings (Text-fig. 5C). There is little doubt that these swellings are homologous with the epidermal ampullae already seen in the first blastozooid and the oozooid. In the zooid of *Distaplia bermudensis* illustrated in Text-fig. 4A the branchial siphon is simple and circular, but the atrial siphon is greatly hypertrophied, though more as an aperture than a siphon.

The enlargement has a double effect. The aperture itself extends posteriorly to the level of the lowest row of stigmata, and ventrally, on one side, it cuts back almost to the endostyle, thereby exposing a large part of the inner branchial sac. The upper or anterior margin of the siphon, in place of being cut back like the lateral margins, extends out dorsally from the thorax in the form of a large and long tapering languet (Text-fig. 4B). This is in marked contrast to the condition in the oozooid and first blastozooid of *D. rosea* (and there is evidence that the oozooid of *D. bermudensis* is similar), in which the atrial siphon is small and circular like the branchial siphon. Smaller zooids from the same colony, however, possessed an atrial siphon like that shown in Text-fig. 4C, with the upper margin of the siphon just starting to protrude. The presence and shape of the languet is correlated with the presence and form of zooid systems in mature colonies. Looking at the upper surface of a colony the zooids are seen to be arranged in more or less star-shaped systems of about six zooids each. All the languets of one system of zooids point to the centre of the system and together form the margin of a common cloacal aperture (Text-fig. 4D).

The heart and gonads lie on opposite sides of the gut loop below the stomach (Text-fig. 4E). The lower end of the heart opens into one of the two vascular epidermal tubes already mentioned. The other tube opens into lacunae towards the stomach and gonads.

The zooid shown in Text-fig. 4A is clearly a sexually mature form with a rosette of testicular follicles and a sperm duct congested with sperm. Growing ova of various sizes lie at the centre of the male follicles. The sexual maturity of the zooid, however, refers only to the male component, and zooids of this species attain male and female phases at different times. Large zooids, when newly functioning, have fully matured testes and full sperm duct. Only as growth proceeds do they acquire mature ovaries and oviduct.

Following a rather small but appreciable amount of growth, the largest ovum attains its final and maximum size. Coinciding with this the brood-pouch appears for the first time as a small pear-shaped protrusion of the body-wall near the base of the dorsal atrial cavity (Text-fig. 5A). The nature of the brood-pouch was examined by Bancroft (1899), who first figured it in its early stage of development. He recognized that the epidermal sac contained an inner tube doubled upon itself and continuous at one end with the oviduct and at the other with the atrial cavity, but was unable to determine to what extent oviduct and atrium each contributed to the pouch. He was, however,
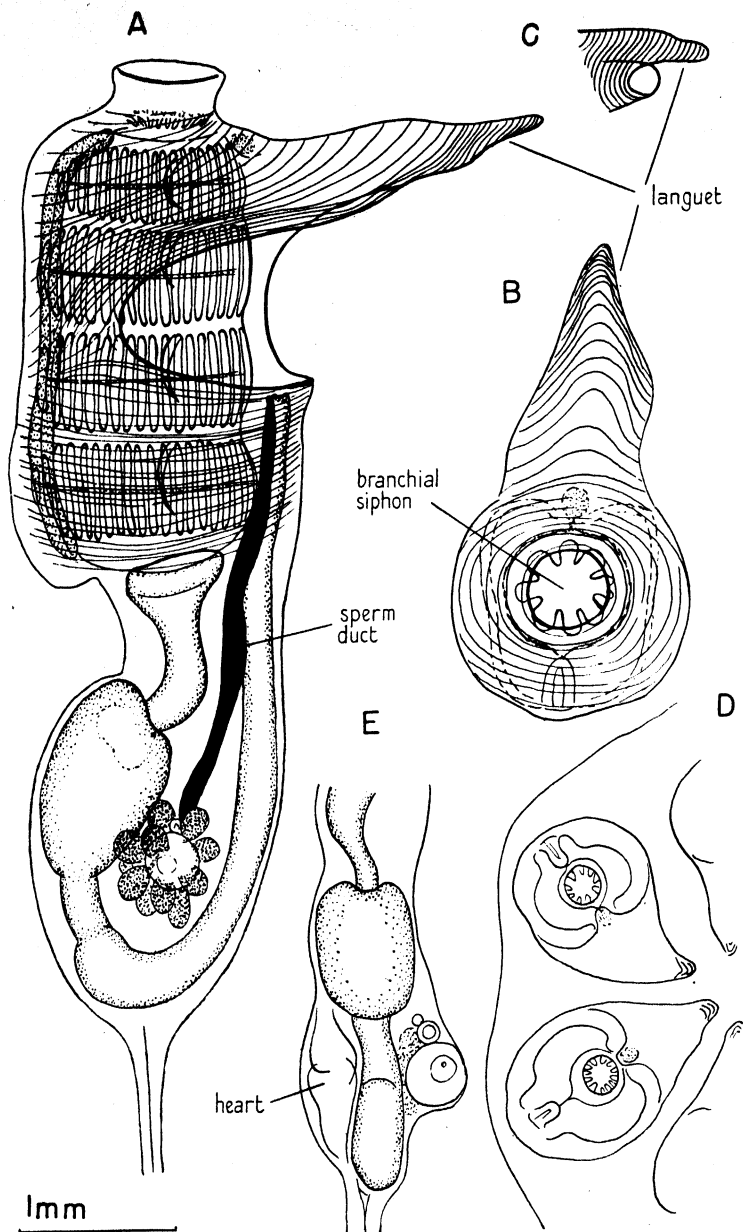
TEXT-FIG. 4. *D. bermudensis*. A. Mature zooid in primary, male, phase, showing enormous gape of the atrial siphon and the growth of the dorsal rim as an atrial languet. B. Dorsal view of branchial siphon and atrial languet. C. Atrial siphon from younger zooid at onset of local atrial growth. D. Part of a zooid system to show convergence of atrial processes toward common cloacal aperture. E. Ventral view of abdomen showing heart and gonads on opposite sides of the intestinal loop.

able to account for the fact that advanced brood-pouches contained embryos arranged so that the youngest was at the bottom and the oldest at the top. He saw accurately (in *Distaplia occidentalis*) that eggs pass up the oviduct and down a narrow descending duct to the base of the pouch, subsequent eggs pushing the first ones up the ascending part of the double tube. This account is fully confirmed for the present species. The condition, however, can be further clarified, and is illustrated in Text-fig. 5B. The sperm duct is not involved in the least in the formation of the brood-pouch, but persists as a duct closely applied to the rectal intestine and opens alongside the anus into the atrial cavity. The oviduct, on the other hand, grows distally in such a way that the distal end remains opening into the base of the atrial cavity while immediately posterior to this it bulges out as a loop, adjacent epidermis accommodating to it. The loop consists of a narrow descending limb of a diameter no wider than the unmodified oviduct, and a much wider ascending limb. Long before the first egg passes up the oviduct proper and into the pouch, considerable masses of spermatozoa can be seen as a diffuse column within the pouch, having entered through the atrial oviducal opening (Text-fig. 5B). Eggs subsequently pass up the oviduct and down the descending limb in the pouch. In so doing they become compressed and sausage-shaped, regaining the typical spherical state upon emergence into the base of the wide ascending limb. Each egg passes up and enters the bottom of the pouch in this manner as maturation is attained, and a series of perfectly graded embryos from early cleavage to mature tadpole stage are usually found in well-developed pouches. The pouch continues to grow as the eggs successively reach it, until a characteristic maximum length is reached. Finally, even though there may still be growing ova in the ovary, the neck of the pouch becomes greatly attenuated and constricts off from the body of the parent (Text-fig. 5C). The elongated brood-pouch thus becomes isolated from the zooid and in fact persists with its contained series of developing embryos usually long after the dissolution of the zooid that originally produced it.

The isolated and abandoned brood-pouches with their embryos and tadpoles are said to migrate through the matrix of the colony to liberate the larvae as they become ready. A mechanism for such a migration is difficult to imagine, and in fact there is no migration in the sense implied. The same result is reached by a progressive sloughing off of the surface test material of the colony until the anterior ends of the pouches come to lie at the surface. This is an inevitable result of the dissolution of the parent zooids. The test matrix is a complex medium and is one that needs to be actively maintained by adjacent living tissues. With the reduction of the zooids, the superficial layers of test soften and degenerate, and the colony surface is in effect cut back to the level of the pouches. While sloughing at the surface does bring the pouches close to that region, some migration is not excluded, for a mechanism does not need to be understood in order to exist. In the case of buds arising from some source deep within the colony, migration towards the surface and orientation there to form systems undoubtedly occurs.
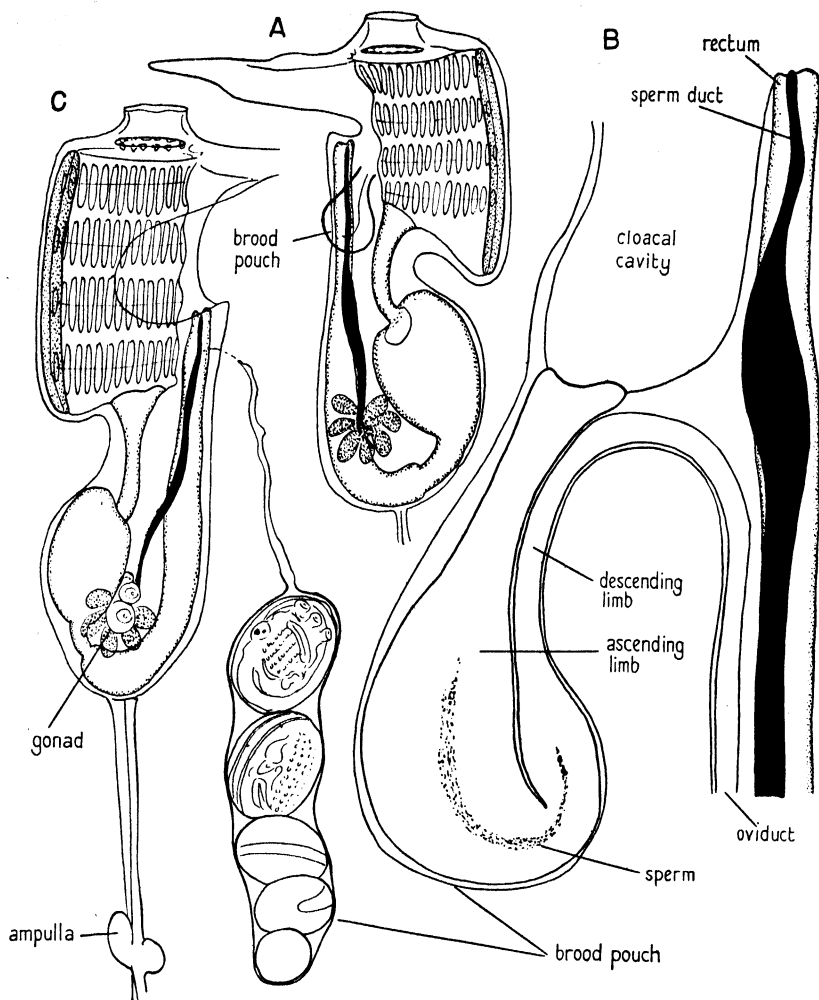
TEXT-FIG. 5. *D. bermudensis.* A. Mature zooid at onset of female or hermaphrodite phase with brood-pouch at early developmental stage. B. Enlarged view of young brood-pouch to show its origin as a bulging local extension of the distal oviduct. C. Mature zooid in final phase with brood-pouch filled with graded series of developing embryos, the neck or stalk of the pouch constricting off from parent at base.

## Growth of Buds

The gross development and growth of the buds is described here in order to complete the growth-cycle of the zooid, leaving the question of early morphogenesis until after the problem of bud origins has been discussed.

Text-fig. 6 shows the growth of the bud from the time shortly after its origin, when it consists essentially of a spherical envelope of epidermis enclosing a more or less spherical inner mass of cells, until shortly before it attains functional activity with siphons opening to the surface (Text-fig. 6E and F). In a quantitative sense, the amount of growth is immense and raises the question of nutrition. The bud from the start is an isolated unit within the matrix of the colony and obviously possesses but a fraction of the substance contained in the developed zooid. The only possible source of nourishment is the surrounding more or less fluid organic medium. There is no accumulation of reserve cells within the bud, no migration of food-laden cells into the bud system during its development, and no connexions made subsequently between the closed bud system and that of any more developed zooids. In any well-established colony degenerated zooids or their dissolution products are seen together with functional zooids, and a rich growth medium undoubtedly exists. That such is the source is further shown by the fact that the first blastozooid of *D. rosea* remains virtually in a state of arrested development all through the functional period of the oozooid, undergoing a rapid growth simultaneously with the dissolution of the oozooid and the consequent availability of its disintegration products.

The primary bud sphere elongates from the first and, at the stage and size shown in Text-fig. 6B, possesses the basic organization of the zooid, with branchial and atrial divisions, endostyle, neural complex, heart, digestive loop, and epicardium. Further development is mainly an elaboration of these parts. The four rows of stigmata are imperforate but are discernible in the stage of Text-fig. 6C. At this stage the abdominal epidermis has extended posteriorly as a long tubular outgrowth with a tendency to form terminal ampullae, anastomoses, and ampullary swellings close to the base of the abdomen proper.

In the next stage (Text-fig. 6D) the stigmata have perforated, and the heart is already beating even though the zooid as a whole is far from a functional state or size. Three features are outstanding. There is an enormous elongation of the pair of posterior vascular epidermal vessels, an elongation which in itself tends to drive the zooid up towards the colony surface, and a sharper differentiation of the cross-connecting ampullae. The gonad is clearly visible as a relatively large mass situated within the intestinal loop. Both branchial and atrial siphons are clearly formed though not open, and the muscle bands of the body-wall are readily seen. At this stage the atrial siphon has a small circular aperture exactly like that of the branchial siphon. In the final stage, shown in dorsal and lateral view in Text-fig. 6E and F, there is a much larger size but little structure change. The gonad is more clearly seen to be testis only, while, right up to functional activity at least, the atrial siphon remains small and circular, with no resemblance to the wide-gaping siphon of the mature zooid with its long languet. The posterior growth of the vascular vessels is due, for the most part at least, to continuous terminal proliferation of the distal extremities, and is doubtfully an example of true relative growth.
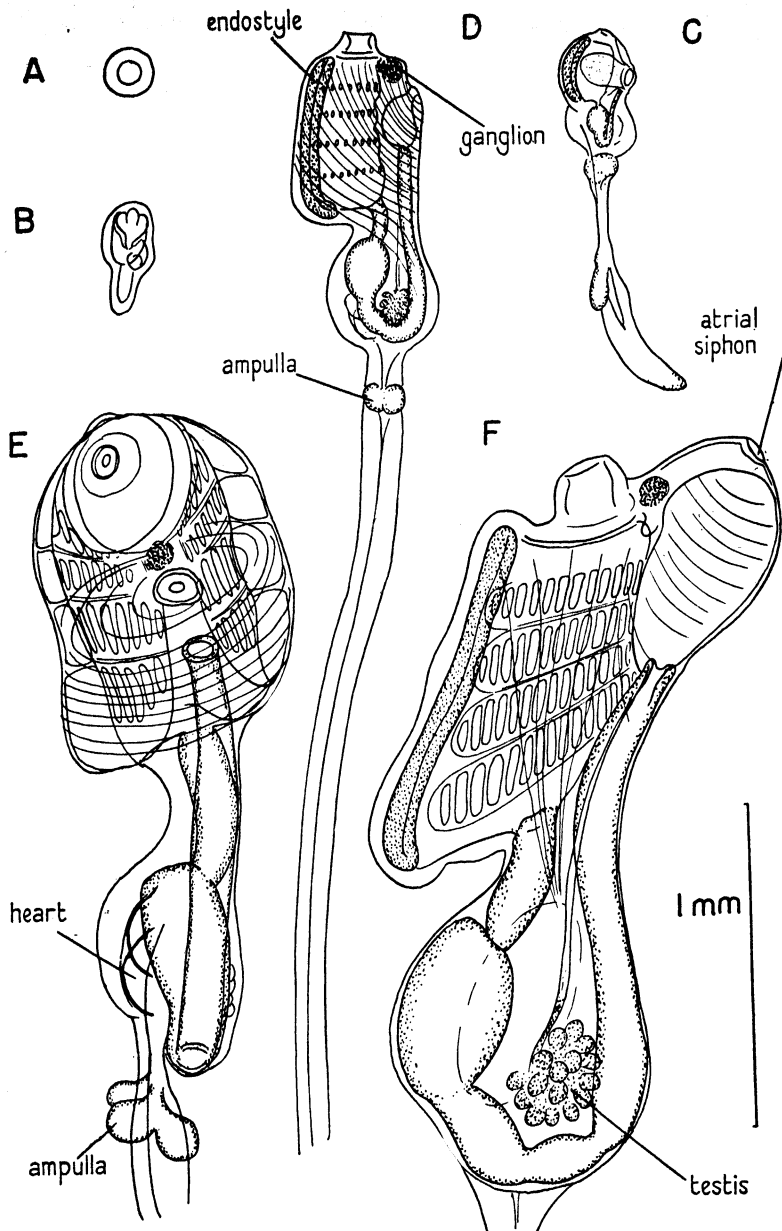
TEXT-FIG. 6. Gross development of a bud (*D. bermudensis*). A. Bud at initial size and form. B. Bud with essential morphogenesis complete. C. Stage with stigmata represented at 4 imperforate bands. D. Stigmata well perforated, testes well developed, and vascular stolon enormously elongated. E, F. Dorsal and lateral views of advanced stage, showing small circular atrial siphon.

With regard to the rest of the developing zooid, there appears to be little significant change in body proportions between the stages shown in Text-fig. 6B and F; in other words, during this phase and excluding the vascular stolon, growth of the whole seems to be a unitary process with all organs and tissues growing at the same pace.

With the attainment of functional activity by the zooid the siphon begins to exhibit relative growth culminating in the form of siphon already described for the mature adult. It is as definite an example of relative growth as any, and implies a sharp change in the developmental system at the time of zooid functioning, from a closed morphogenetic system to an open one.

*Gonad formation in* D. bermudensis, D. rosea, *and* D. magnilarva

Leaving aside for the present the origin and nature of the gonad Anlage, the gonad as initially discernible in whole mounts develops in the same manner as the rest of the zooid, namely, as a growing mass of reproductive tissue exhibiting no further change except for size throughout the developmental period up to the functioning of the zooid, after which relative changes occur.

The condition in zooids of *D. bermudensis* at the end of bud development is shown in Text-fig. 7A, and this is the condition of the gonad even in the youngest stages. It grows without change until the zooid as a whole becomes functional, after which the largest ovum rapidly grows to its maximum (Text-fig. 7A, B, C), successively followed by several others. While clearly hermaphrodite, the zooids are at first functional males and only later function as females, so that finally only female structure may be visible.

In *D. rosea* the youngest buds are seen to contain gonads in which both male and female components are equally represented. The condition at the time of initial functioning of the zooid is shown in Text-fig. 7D, testicular follicles converging to the base of the sperm duct, while the ovary extends from the body-wall in a pear-shaped sac.

*D. magnilarva* is of greater interest. Uljanin (1885), studying live colonies at Naples, concluded that not only were all the zooids of any one colony all of the same sex, either male or female but never hermaphrodite, but that there was a regular alternation from one sex to the other. Caullery (1895) confirmed Uljanin that colonies were unisexual, but considered young buds (0·8 mm. long) to be hermaphrodites, changing into unisexual adult zooids (6·0 mm. long) through a regression of one sexual component on the other at a relatively early stage, whichever happened to be the smaller.

In the only colony of *D. magnilarva* examined here, very large and obtained from Naples, the great majority of the zooids were purely female with large ovaries and brood-pouches with many embryos. Very young buds (0·7 mm.) in the vicinity of mature female zooids showed only developing ova (Text-fig. 7E). In one region of the colony, however, about two dozen zooids had no brood-pouches, no sign whatever of ovaries, but large well-developed rosettes of testis. In their vicinity the smallest buds appeared to possess only
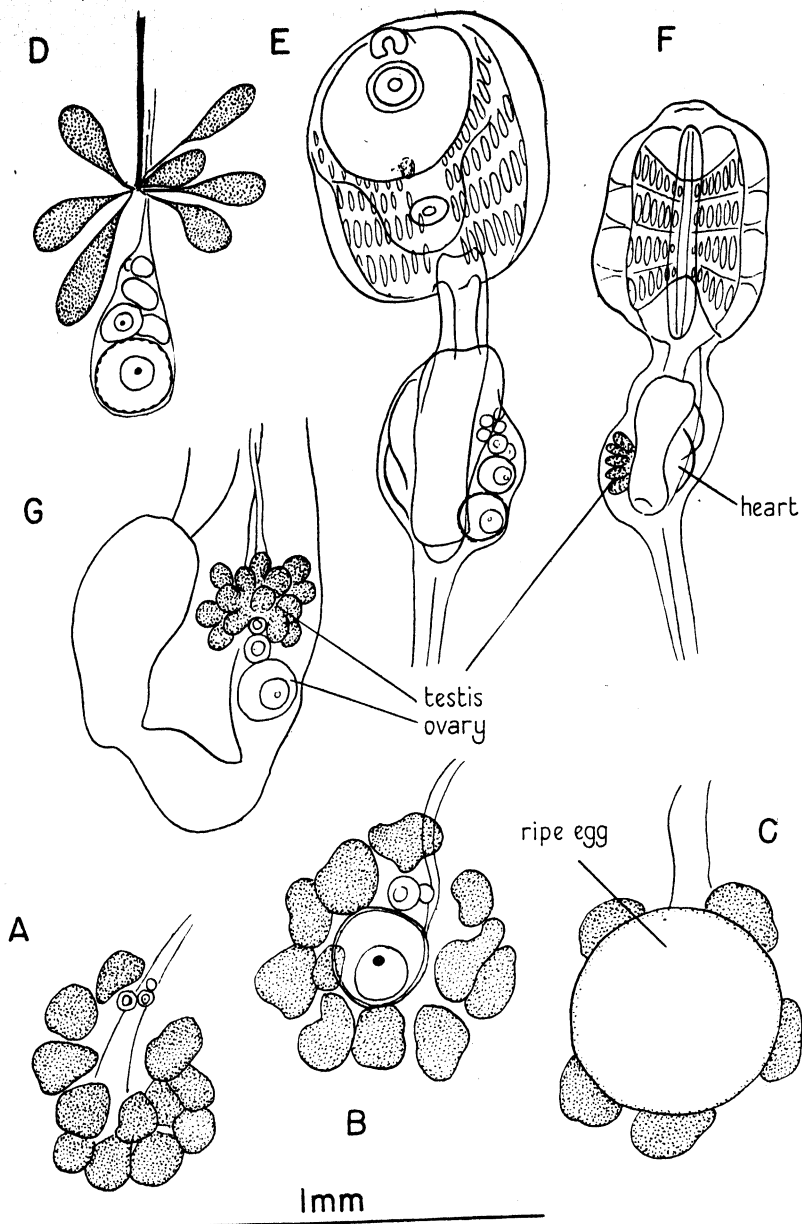
TEXT-FIG. 7. Gonad development in various species of *Distaplia*. A, B, C. Stages in gonad development of *D. bermudensis* from initial functioning of mature zooid to formation of first ripe ovum. D. Gonad of *D. rosea* in developing bud. E, F. Young buds of *D. magnilarva* showing female and male types respectively. G. Occasional hermaphrodite gonad in young *D. magnilarva* bud.

developing testes (Text-fig. 7F). In a few young buds in different parts of the colony, however, hermaphrodite gonads (Text-fig. 7G) were encountered.

These conditions raise interesting questions concerning the general mechanisms of sex determination, and will be further discussed following an analysis of the origin and nature of gonad Anlagen in buds too small to be
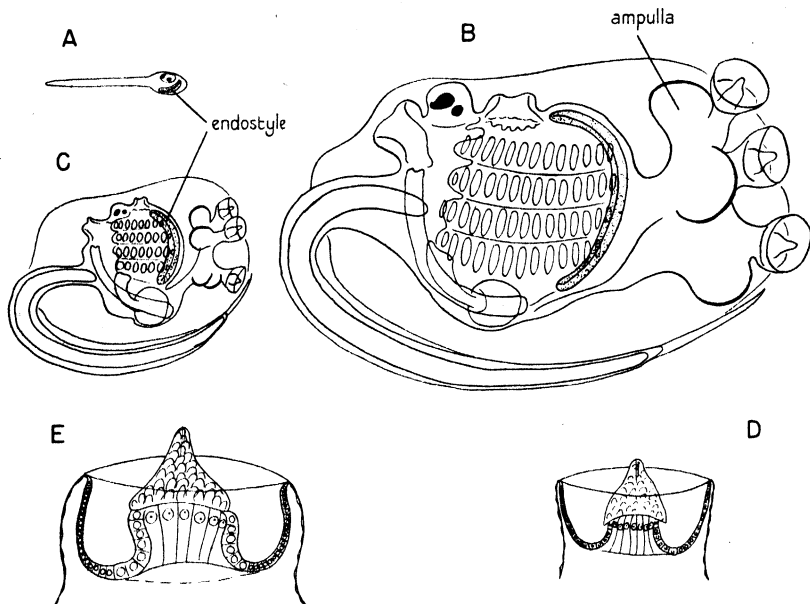


TEXT-FIG. 8. Size and organization in *Distaplia* tadpoles. A. Tadpole of *Ascidia* for scale comparison. B. Tadpole of *D. magnilarva*. C. Tadpole of *D. rosea*. D, E. Adhesive organs of *D. magnilarva* and *D. rosea* drawn to same enlarged scale, showing some cell numbers.

studied except by serial section. Functional unisexualism is found elsewhere among ascidians only in species of *Colella*, a genus closely related to *Distaplia*.

## PART II. THE DEVELOPMENT AND NATURE OF THE TADPOLE LARVA

The embryonic development of *Distaplia* has been described at length by Dawidoff (1889), but at a time when little or nothing was known concerning the distinctive cleavage patterns of the ascidian egg in other forms, and when the main interest lay in the general chordate character of development and its relation to the ascidian organization subsequently formed. The nature of the tadpole as a functioning organism was neglected, while the pattern of early cleavage was overlooked. The present account is therefore mainly of these two phases and does not emphasize the intervening span of development to which Dawidoff gave adequate attention.

As an introduction to the nature of the tadpole, Text-fig. 8 illustrates the general form and structure, and particularly comparative sizes. The smallest and largest tadpoles of *Distaplia* species are shown, those of *D. rosea* and *D. magnilarva*. Those of the other species studied, namely, *D. bermudensis*, *D. garstangi*, *D. clavata*, and *D. occidentalis*, are intermediate in size between these two extremes. A tadpole of *Ascidia* is shown at the same scale. It is typical in size for all oviparously developing ascidians, whatever the family. It is clear, however, that variation in the size of the *Distaplia* tadpoles does not significantly affect their organization. The relative shortness of the tail of *D. magnilarva* tadpoles is in part due to the fact that the tail is composed of comparatively solid tissue, whereas the trunk and anterior structures consist virtually of 2-dimensional sheets of tissue, and a difference in egg diameter therefore expresses itself more strikingly in the trunk than the tail. The sensory organs differ in size but not in number or type of cellular components, while in the case of the adhesive organs (Text-fig. 8D, E) the size of the cells varies but not the number or organization. In contrast, however, multiple structures of the permanent ascidian organization, i.e. gill slits, do vary in number approximately with the size of the whole, the constituent cells varying in number but not size.

### Cleavage

In most species of *Distaplia*, eggs in process of early cleavage are rarely encountered and are in any case difficult to orientate, the more so when they lie somewhat congested at the bottom of a brood-pouch filled with developing embryos at all stages. The present account is based on colonies of *D. clavata* collected in July by M. J. Dunbar at Ungava Bay. They are peculiar in that all are almost at the same stage, with young brood-pouches containing either one or two eggs in the earliest stages of cleavage, and, from these abundant examples, the patterns could be readily observed; they are illustrated in Text-fig. 9.

In Text-fig. 9A a complete young pouch is shown with both limbs of the pouch indicated. The contained egg, in the 2-cell stage, is enclosed in a chorion typical of viviparous forms with follicle cells flattened out over its surface. Both of the first two cleavages pass through the animal pole as indicated by the position of the polar body. The third cleavage is equatorial, and, in spite of the large size and comparative yolkiness of the egg, produces a bilaterally arranged group of 4 on 4 cells as described for the small egg of *Styela* by Conklin (1905). This stage and its imminent transition to the 16-cell stage is shown in Text-fig. 9C and D. The 16-cell stage proper is shown from lateral, posterior, and dorsal sides in Text-fig. 9E, F, and G respectively. The characteristic bilateral pattern is as clearly marked, therefore, in the developing egg of *Distaplia* as it is in any other ascidians. Scott (1946) has shown that the comparably yolky egg of *Amaroucium* is essentially the same, and it follows accordingly that increase in size and yolk content of ascidian eggs in no way significantly affects the course of early development, and the basic developmental patterns persist.

One of the more striking features of later development is the great increase in size from the egg to the tadpole (Text-figs. 10–12, especially fig. 10A–E). While these illustrations are of *D. bermudensis*, the expansion is typical of all species of the genus and is mainly a reflection of the large amount of yolk within the egg. Text-fig. 10B and C represent young embryos showing two
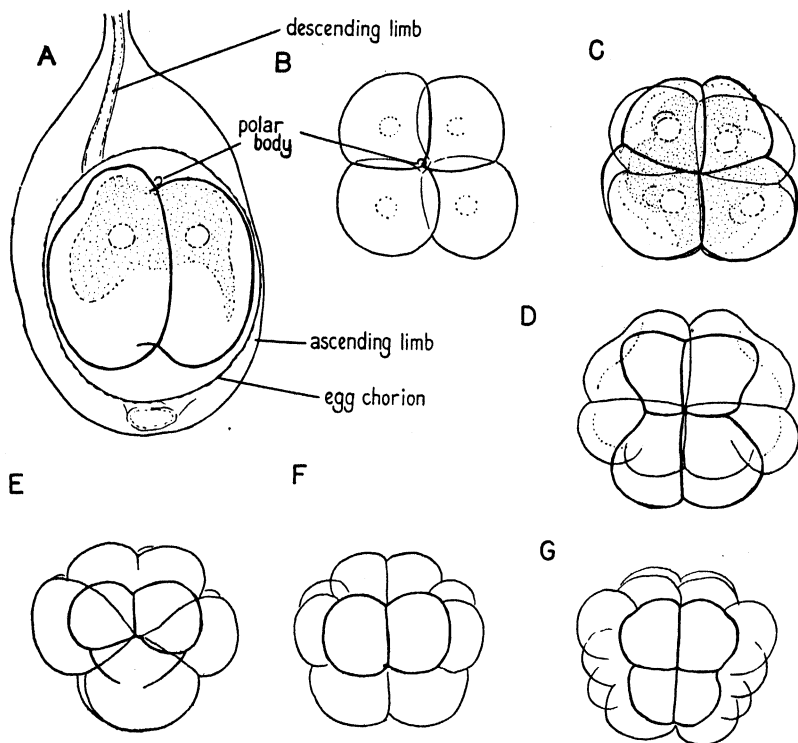


TEXT-FIG. 9. Cleavage stages of *D. clavata*. A. 2-cell stage within brood-pouch. B. 4-cell stage. C. 8-cell stage. D. 8-cell dividing to 16-cell. E, F, G. Three aspects of 16-cell stage.

stages in the formation of the notochord. Approximately 40 notochord cells are produced, as in all other ascidians, and the tail grows out under the pressure of the swelling cells, at first as two interdigitating rows (Text-fig. 10B) and then as a single column of 40 (Text-fig. 10C). In this last stage the tail is shown bent over so that the tail muscle-cells are seen in optical section around the notochord. These are also now present in the full final number. Mesenchyme cells also appear in large numbers, between the epidermis and endoderm. Succeeding stages show a rapid overall expansion, the epidermis and endodermal envelopes becoming widely separated from one another and the tail with its enclosed notochord extending around the full circle of the

enlarging trunk (Text-fig. 10D and E). In the stage shown in Text-fig. 10D, the larval sensory vesicle exhibits the first sign of pigmentation of the sensory cells forming the ocellus and otolith, while the three anterior adhesive organs
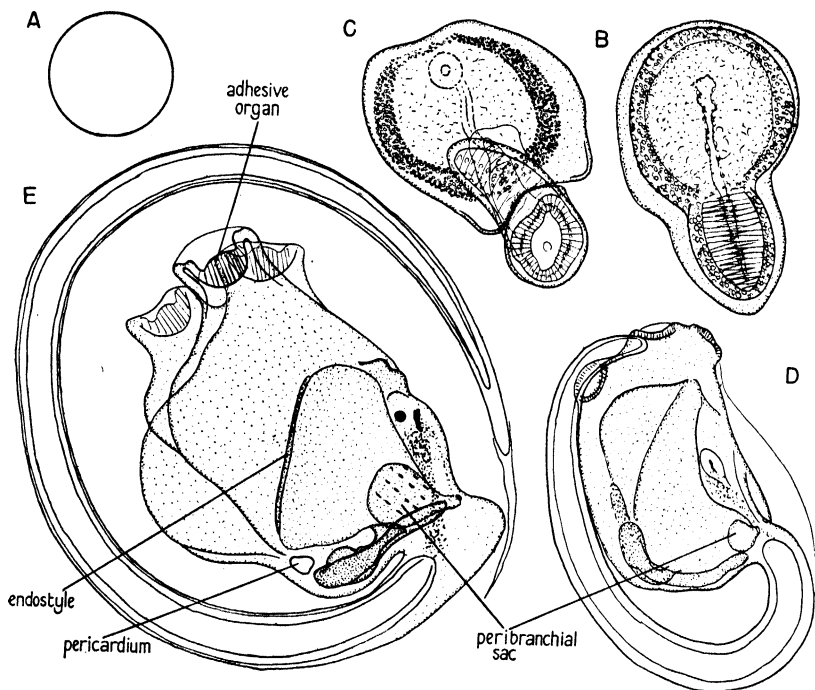


TEXT-FIG. 10. Development of the embryo (*D. bermudensis*), to same scale as tadpole stages in Text-figs. 11 and 12. A. Egg outline for size comparison. B. Embryo with trunk and tail differentiated, full number of notochord cells delimited. C. Somewhat older embryo with branchial siphon forming, and tail recurved showing attainment of full number of tail muscle-cells in optical section. D. Young tadpole form with first trace of sensory pigment, peribranchial sac, and rudiments of adhesive organs. E. Later stage, with stigmata appearing and pericardium formed.

are evident as shallow cups of thickened epidermis. At the same time the peribranchial sacs appear as a pair of dorso-lateral invaginations.

The later stage (Text-fig. 10E) shows all developmental units already present, with four rows of dividing stigmata visible in the now-conjoined peribranchial sacs, a digestive loop with oesophageal, stomach, and rectal divisions, a completely segregated heart, distinguishable endostyle, &c. The adhesive organs each are differentiated into a central mass of columnar cells and a marginal cup wall giving a sucker appearance to the whole.

The two stages illustrated in Text-fig. 11 are even more recognizable as tadpoles, although they do not represent the active free-swimming stage. The

main interest in these stages, taken together with the final active stage (Text-fig. 12), lies less in the continued differentiation of structures and organs than in the relative growth or form changes occurring in the anterior trunk, the branchial wall, and the distal abdominal region. While each progressively changing process is a phenomenon apparently unrelated to the others, there is equally a rigid correlation in a time sense, so that a knowledge of the stage reached in any one case accurately indicates the state attained by the other two. Since these are, however, essentially growth activities, each is best described in terms of the three stages illustrated without much reference to the stage as such.

*Branchial Wall*

The branchial wall is essentially the area of the combined peribranchial and endodermal layers. The peribranchial sacs are ectodermal invaginations on both sides, almost at once fusing mid-dorsally to form the atrial siphon, and coming to lie with the inner side of the sac in contact with the outer surface of the endodermal pharynx. For a while the rapidly expanding sacs appear to slide over the endodermal wall, but this may not be actually the case. When the sac has spread about one-third the way across the endodermal wall toward the endostyle, four rows of thickened tissue appear, the four rows of stigmata, at first imperforate but quickly breaking up into separate perforate units. As the peribranchial sac appears to spread toward the endostyle anteriorly, and also dorso-ventrally, the stigmata increase in number to about twice that originally recognizable, until each row consists of about a dozen well-formed gill slits (Text-figs. 11 and 12). The reason for doubting that the peribranchial wall is sliding along the endodermal wall towards the endostyle is that the stigmata are perforate at a relatively early stage in the process, and as perforate structures clearly undergo elongation and division. Since a perforate stigma involves a fusion and perforation of both peribranchial and branchial layers, one of the two layers cannot really be extending relative to the other, and a more reasonable interpretation is that the combined area is growing and differentiating as a unit, while the purely endodermal wall between it and the endostyle becomes correspondingly reduced in some way.

The number of gill slits per row in the tadpole of any species of *Distaplia* is approximately half the number characteristic of the mature adult zooid of each species respectively.

*Anterior Trunk*

As seen in Text-fig. 11A, the anterior half of the trunk is wide and voluminous, with an extensive blastocoelic space within. In the later stage (Text-fig. 11B), four epidermal thickenings associated topographically with the three adhesive organs appear, while a deepening constriction begins on the dorsal side between these structures and the branchial region. This constriction progresses until it effectively cuts back in front of and below the branchial sac, so that the three adhesive organs and four ampullae are in fact connected

A

peribranchial sac

right epicardium

pericardium

left epicardium

probud

B

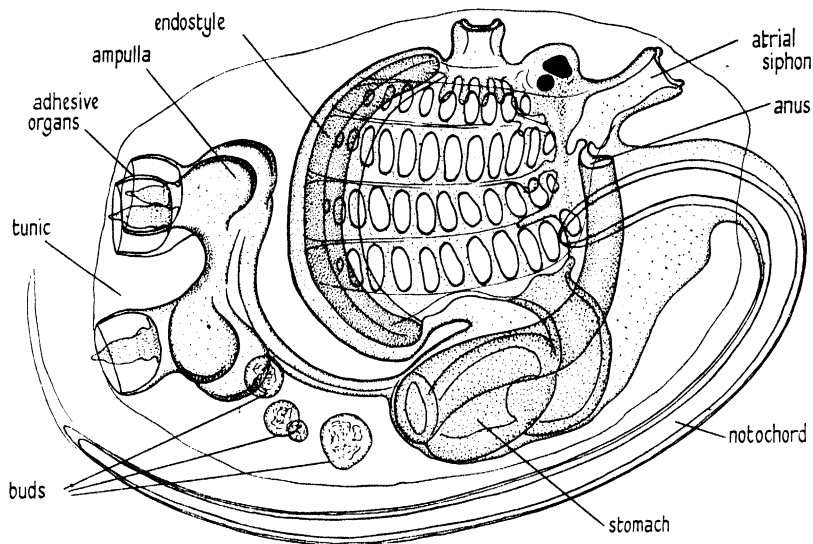probud

pericardium
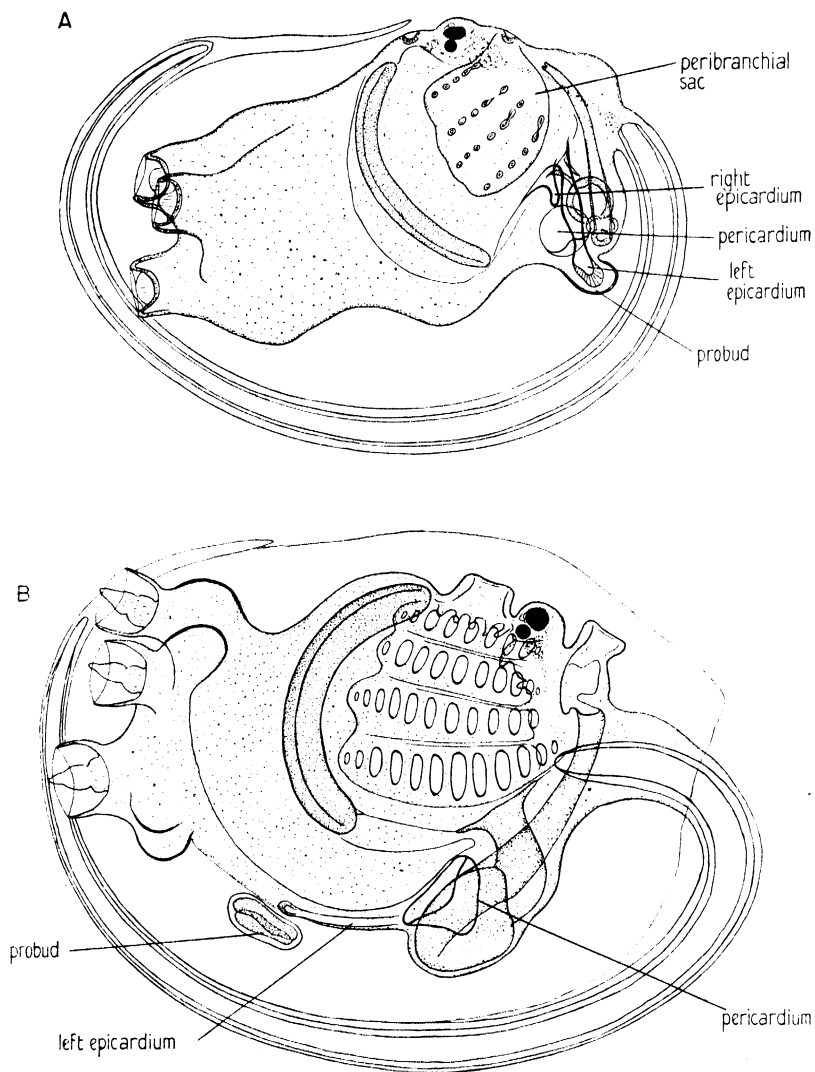
left epicardium

TEXT-FIG. 11. A. Stage with perforating stigmata, showing short right epicardium terminating above the pericardium, and left epicardium protruding ventrally to form the probud. B. Nearly mature tadpole with probud separated and elongating.

with the distal end of the abdomen by a pair of epidermal tubes or vessels (Text-fig. 12). The adhesive organs are transitory organs of the tadpole, but the ampullae and the vessels survive metamorphosis and represent the stolonic vascular vessels and ampullae characteristic of all blastozooids and described in the first section of this paper.

### Bud Formation

The details of bud production and their subsequent history is the subject of the last part of this investigation. In the present instance the interest lies more



TEXT-FIG. 12. Mature tadpole of *D. bermudensis*. Adhesive organs and ampullae connected to trunk by narrow vascular stolon, probud constricted into four definitive buds.

in the relative time at which certain of these events occur. In Text-fig. 10A the epicardium consists of two evaginations from the floor of the pharynx, one short and ending immediately above the pericardial vesicle, the other extending to the ventral abdominal epidermis. This region of the epidermis, together with the distal end of the epicardial tube, bulges out as relatively thickened epithelia from the general contour of the body-wall.

In the succeeding stage (Text-fig. 11B) the protruding region has constricted off and is further elongated as the probud, while the residual epicardial tip, still relatively thick but much narrowed, has extended far anteriorly.

In the final tadpole stage (Text-fig. 12) the probud is segmented into four definite buds, the epicardium meanwhile having withdrawn to its abdominal site.

## Structure of the Active Tadpole

The fully formed tadpole is in a sense a triple organism, consisting of the transient structures of the tadpole as a purely larval organism, the relatively permanent organization of the oozooid, complete except for minor changes in orientation and attachment, and among the buds is the first blastozooid destined to replace the oozooid (Text-fig. 12).

The oozooid organization is virtually complete and within a very few hours of actual functioning. The siphons show some contractility, and the heart beats and exhibits the characteristic reversal of beat from the first, even in the free-swimming tadpole phase. The branchial sac with its four rows of large definitive stigmata, dorsal languets, and branchial tentacles, and the alimentary canal, both appear morphologically complete, though cilia are not active. Anteriorly the ampullae, which will extend from the basal end of the future erect and attached oozooid, are connected to the region of the heart by the double vascular stolon.

The purely tadpole organs consist of three units, the adhesive organs, the sensory vesicle, and the tail, all of which are destroyed during the process of metamorphosis. The free-swimming period is short at the best, rarely more than of 2 or 3 hours' duration, and the habitat of most species is such that there can be little opportunity for selectivity on the part of the tadpole.

### Adhesive Organs

There are three adhesive organs, the typical but not invariable number for ascidian tadpoles, and they are arranged also characteristically with one median and ventral and two dorso-lateral. Each consists of a central adhesive cone of glandular cells secreting the adhesive cement through a narrow tubular core, and a sucker-like cup of non-glandular epidermis surrounding the glandular part. There is apparently no real sucker function, for the cups become comparatively shallow at the time of attachment and seem to serve mainly to confine the spreading viscous secretion of the central structure to an effective area. As in the tadpoles of *D. magnilarva* and *D. garstangi*, in those of *D. bermudensis* the central glandular column of each adhesive organ consists of 6 or 7 high columnar secretory cells.

### Sensory Vesicle

The sensory vesicle forms an obvious protrusion between the branchial and atrial siphons. The general organization is shown in Text-fig. 13. The vesicle and its components vary in size in general proportion with the tadpole as a whole. Otherwise the structures are fairly typical, in kind and arrangement, of those of ascidian tadpoles in general.

A dorsal view of the area between the siphons is shown in Text-fig. 13A, with the sensory vesicle proper lying side by side with the neural gland; the neural ganglion of the oozooid is situated in the midline between the sensory organs on one side and the gland on the other. Text-fig. 13B is a lateral view of the same structures, showing the unicellular nature of the otolith, the

3 lens-cells partly embedded in the pigmented mass of the ocellus. The pigment consists of closely packed granules contained in about a dozen cells arranged in the form of a cup (shown in Text-fig. 13C, an ocellus with pigment partly bleached out).

*The Tail.* The tail as a whole is twisted through 90° relative to the axis of the trunk, so that the cuticular membrane is horizontal.

The notochord consists of approximately 40 cells, as it does in all ascidian tadpoles examined in this respect. The intracellular vacuoles, however, are so enlarged that they have fused end to end to form one long cylindrical tube, with the nuclei compressed and lying in the containing membrane (Text-fig. 13E).

The notochord lies within a tapering epidermal tube of similar shape, but without coming into actual contact with the epidermis. This is especially evident at the tip, where the epidermal envelope is seen to extend a considerable distance beyond the distal end of the notochord.

The tail muscle-tissue consists of two bands, one above and one below the notochord (Text-fig. 13D). The bands adhere closely to the outer surface of the notochord, though they do not extend as far as its distal end (Text-fig. 13E), a condition again shared with most other tadpoles.

The muscle-cells are, however, unusual in two respects. In the tadpoles developing from all small eggs, the number of muscle-cells is as constant as the number of cells forming the notochord and is about 36. In the case of larger eggs, tadpoles developing from them may acquire tail-muscle tissue amounting to several hundred cells, e.g. *Amaroucium, Stolonica.* In *Distaplia bermudensis* the number slightly exceeds 2,000, several times as many as in any other form. The gearing of the development and differentiation of notochord and muscle-bands offers an intriguing comparative study. The second peculiarity is that whereas in most other tadpole types the fused cortical regions of the cells through which the continuous myofibrillae extend lie at the outer periphery of the bands, the fused cortical zones lie on radial axes from the centre of the notochord (Text-fig. 13D). The myofibrillae accordingly pass through the band of tissue only within the mass, and the surfaces that normally contain them are destitute. Whether the arrangement is a direct consequence of the extreme subdivision of the presumptive muscle-tissue and related shift in cell orientation or not, the effect is to produce a greatly extended area of cortical zone and therefore of contractile efficiency.

## PART III. ORIGIN, FORMATION, AND EARLY DEVELOPMENT OF BUDS

All earlier accounts of bud formation in *Distaplia* concern bud production in the tadpole larva, as does part of this section. Efforts have been made previously to find direct evidence of formation of buds by adult zooids, all apparently without success. Julin (1896) concluded that none was formed and the buds seen in the larva gave rise to all subsequent generations of blastozooids. This has been supported by the fact that most observers have reported probuds in process of division in colonies of all ages. Daumezon
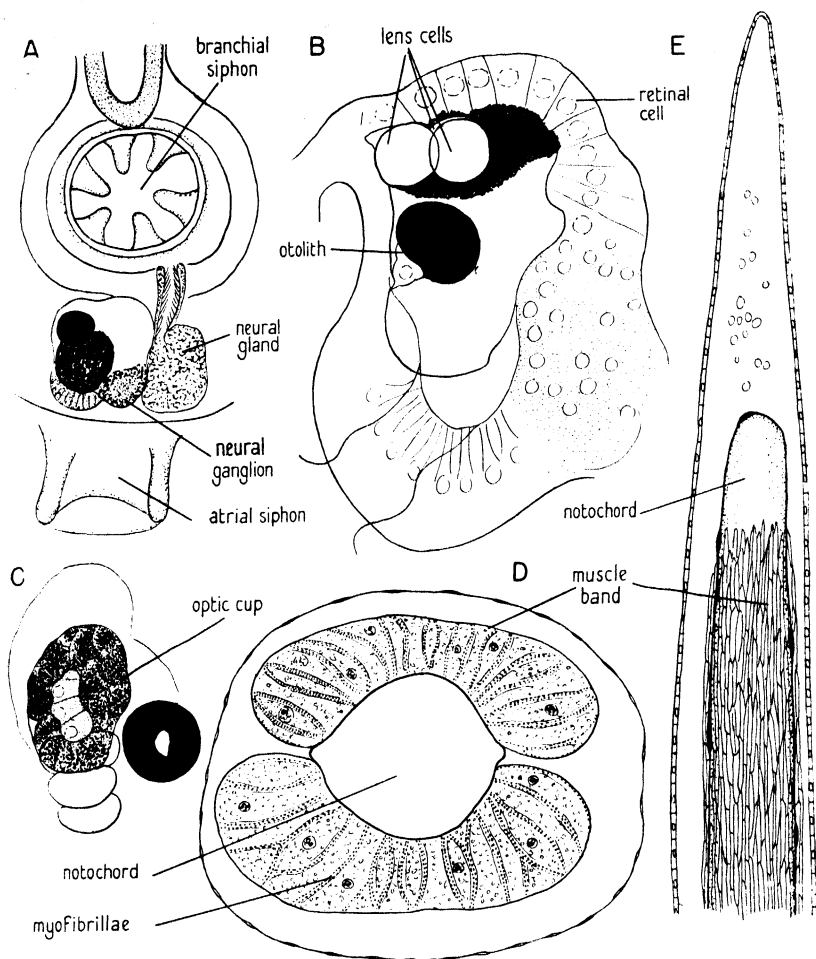
Text-fig. 13. Details of tadpole structure (*D. bermudensis*). A. Dorsal view of sensory vesicle and atrial and branchial siphons. B. Reconstruction of sensory vesicle from adjacent sections showing unicellular otolith, lens cells, retinal cells, and ganglion. C. Dorsal view of partly bleached vesicle with otolith, 3 lens cells, and with centre of optic pigment showing sensory cones. D. Cross-section of tail with completely vacuolate notochord, and muscle-cells arranged with myofibrillae extending only through coextensive surfaces. E. Tip of tail showing relative extensions of muscle-cells, notochord, and epidermal envelope.

(1909), however, challenged Julin's conclusion on purely *a priori* grounds, though Brien (1939) has accepted Julin's opinion wholeheartedly, and in his excellent contribution on the budding and organogenesis of the blastozooid of *D. magnilarva* has limited his investigations to the larval stage, even though

living mature colonies were used to obtain the material. He refers to an earlier statement by Berrill (1935), that buds are produced by blastozooids as well as by larvae, as astonishing. On the assumption, therefore, that the only buds produced are those of the tadpole, Brien is forced into a description of certain related phenomena that appears to be a complete misinterpretation of his observations.

Accordingly the present account consists of a description of the manner in which buds are produced by blastozooids of every generation, a description and interpretation of the probuds formed by both larva and blastozooid, and finally a developmental analysis of the peculiar sexual conditions referred to in the first section.

### Origin of Buds from Blastozooids

The failure of previous investigations to demonstrate bud formation by the blastozooids of established colonies has been for the most part due to the nature of the colonies of the two species examined, namely, *D. magnilarva* and *D. rosea*. In the large colonies of *D. magnilarva* buds at all stages of development are generally to be found, and probuds in process of division usually encountered. It has been almost impossible to relate even the smallest buds with any adjacent structures, although Salfi (1925) suggested the vascular stolons as a source, an opinion rejected by Brien (1939). It should be emphasized that buds at their earliest recognizable stage are of a size comparable with the innumerable trophocytes scattered through the colony matrix, and that nutrition of the developing bud is maintained without contact with any other organized structure.

Colonies of *D. rosea* are comparatively small and each colony head consists usually of no more than a dozen zooids. Many such colonies have been sectioned entirely and it is possible to correlate the stages attained by co-existing generations. These observations may be summarized as follows. Very young buds, not probuds, are not invariably present. In most cases only developing buds with a recognizable degree of morphogenesis are to be seen coinciding with the presence of functional zooids. Primary buds are present in numbers only in colonies in which the adult zooids are degenerate except for persisting brood-sacs with embryos. If buds are produced by blastozooids, the evidence suggests an origin at the end of the individual blastozooid growth cycle. Likewise the segregative process must be of brief duration or buds in actual formation should have been seen.

With these tentative conclusions in mind, the large colonies of *D. bermudensis* were cut into small pieces, each piece including zooids near the surface and the matrix below them for the full thickness of the colony. In any one region zooids were found to be more or less at the same stage, newly functional with testis alone well developed, larger individuals with brood-pouches, or undergoing regression. Each stage was examined, from young developing buds through all functional stages up to and including the regressing zooids as long as anything could be recognized as having been a zooid.

No evidence of bud formation was observed in any of the developing or active stages. When it came to the study of regressing zooids the results were somewhat unexpected. Judging by the comparatively small number of zooids
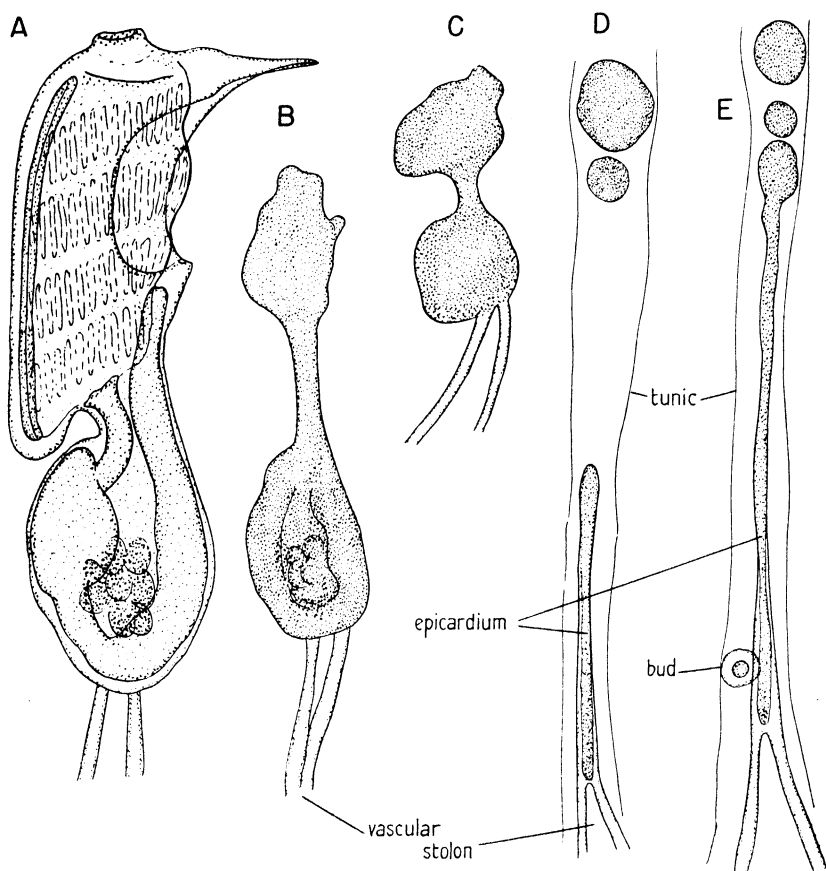


TEXT-FIG. 14. A, B, C. Stages in degeneration of mature zooid of *D. bermudensis*. D. All but final stage, with zooid reduced to minimum but epicardium surviving and extending. E. Similar stage to D, with bud formed near posterior tip of epicardium and lying within stolon tunic limits.

found undergoing regression, it is probable that the whole process is relatively brief compared with the growth cycle as a whole. Text-fig. 14A, B, and C show three early stages of regression, drawn to the same scale as the mature zooids illustrated in Text-fig. 4. The histological details of regression are not relevant here, and have been well worked out by Caullery (1895). In effect, however, the whole differentiated structure of the zooid progressively contracts as tissues shrink, cells autolyse, and phagocytosis ensues, usually

leaving finally one or two small residual masses (Text-fig. 14D), surrounded by heavily food-laden trophocytes. The important fact is that the dissolution process involves the thorax and abdomen but permits the survival of posterior structures. If the regression stages shown in Text-fig. 14C and D are compared, three features become evident. There is continued regression of the thorax and abdomen. There is survival, on the other hand, of the pair of proximal vascular epidermal vessels, while part of the original epidermal wall of the abdomen, together with all or part of the epicardium, not only survives but becomes markedly lengthened. This extension is much greater in the somewhat later stage shown in Text-fig. 14E, and consists of the region occupied by the epicardium, so that the crotch of the vascular processes becomes pushed progressively posteriorly. A comparable extension of the abdomen or post-abdomen, as the case may be, is characteristic of such synoicids as *Aplidium*, *Amaroucium*, and *Morchellium*, differing mainly in the survival in these forms of most of the contained structures and organs. In the last stage (Text-fig. 14E) a small bud is visible near the posterior tip of the epicardium, situated within the limits of the clear test material secreted by the zooid or its remnants. This inclusion in itself suggests a close association of the two, the bud and the epidermal tube with its contained epicardium.

An enlarged view of the same bud region is shown in Text-fig. 15A. All the tissues are clearly in a healthy state as indicated by the clear new tunicin secreted around the whole cylindrical system. The lower end of the epicardium extends to the fork where the two epidermal vessels divide, and is heavily congested with cells derived from its own wall, the epicardium being a completely closed sac or tube. Close to one side of the tip of the epicardium, within the tunicin sheath but external to the epidermis, lies the bud. It is a bud in its simplest shape and smallest dimension, and consists of an outer envelope of flattened epidermal cells similar to that of the adjacent zooid epidermis, and an inner more or less solid mass of cells identical in size and appearance with those of congested epicardial tip. A second example from the same region of the colony is shown in Text-fig. 15B, with a slightly smaller bud already isolated and the lower end of the epicardium turned up together with its enveloping epidermis. The upturning would be the inevitable result of internal extension of the epicardium beyond the fork of the epidermis. The upturned part survives, either as two or three buds successively constricted off, or by estrangulation at its base with constriction to form spheres occurring a little later. Several dozen cases similar to those illustrated were discovered and there seems to be little doubt that this is the normal manner of bud formation in *D. bermudensis*. The examples shown in Text-fig. 15C and D were found previously in a large colony of *D. magnilarva* but not fully understood at the time. Of those that are shown in Text-fig. 15, C is quite evidently the same event, namely, the turning up and subsequent constriction off of the lower end of the epicardium and adjacent epidermis. In Text-fig. 15D the constricted part appears lying in contact with the epidermal tube from which it in part arose, the non-turned part of the epicardium having disappeared,

and the turned-up part having undergone significant growth and differentiation.

There is little doubt that the process of turning up occupies a comparatively insignificant period of the whole growth cycle of the zooid, and the constriction off of this part an even shorter period. This brief event in the cycle, taken together with the fact that only a few regressing zooids can generally be found in any one colony at one time, undoubtedly accounts for its having been previously overlooked.
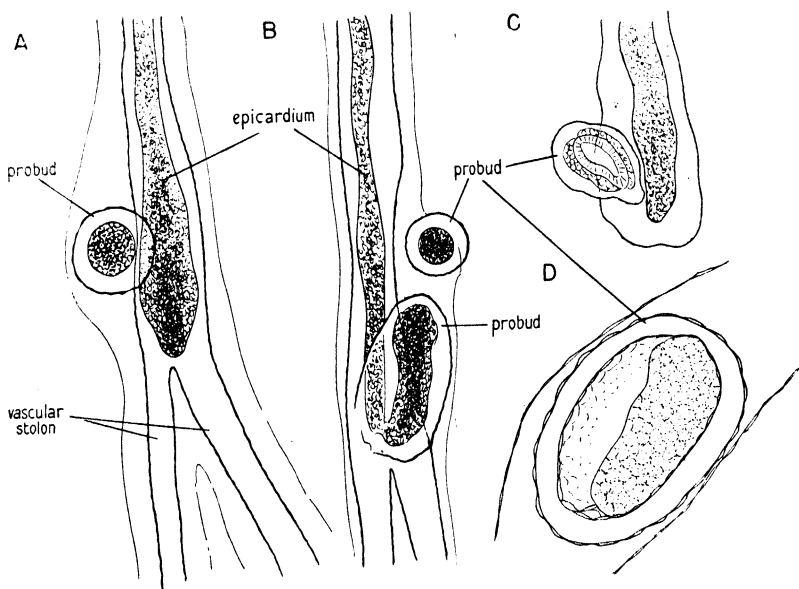


TEXT-FIG. 15. Bud formation from surviving epicardial tip of mature blastozooid. A. Enlarged view of stage shown in Text-fig. 14E. B. Bud constricted from recurved tip of epicardium, with second bud forming. C, D. Probuds just isolated from parent epidermis. (A, B, *D. bermudensis*; C, D, *D. magnilarva*.)

The observations, however, are given additional plausibility in so far as the process just described is essentially the same as that described by many authors for bud formation in the tadpole larva. The difference is mainly in the time at which the process occurs, in the larva when the oozooid organization is elaborating and not yet functional, in the blastozooid only at the termination of the entire growth cycle. This difference may well be due to the difference in manner of development of the epicardium in the embryo and blastozooid, a possibility that will be discussed later, following a more detailed comparison between the two methods.

In Text-fig. 16 are shown six stages in the production of buds by the tadpole larva of *D. bermudensis*. For correlation with specific stages of the developing

tadpole as such, reference is made to Text-figs. 11 and 12. Bud formation occurs at the time the epicardia are growing as tubular structures evaginated from the floor of the pharynx. As the left epicardial sac grows ventrally to impinge upon the abdominal epidermis, the terminal epicardial epithelium thickens and proliferates, the adjacent epidermis doing the same, at least to the extent of conforming to the shape of the inner growing tissue.

The first stage (Text-fig. 16A) consists of a hemispherical abdominal bulge involving the two tissue layers just described. An epidermal constriction separates off the greater part of the thickened epicardial tissue within the bulge to form an inner vesicle of cells derived from the epicardium and an outer one from the epidermis (Text-fig. 16B). Brien has shown that in *D. magnilarva* the constriction itself is due entirely to the action of the epidermis, the epicardium being purely passive in this respect. This has been confirmed for *D. bermudensis* as well, and also in *D. magnilarva* itself.

The double-walled sphere thus produced is, however, a pro-bud, that is, it is not as destined to give rise in its entirety to a single blastozooid, but to several true buds.

After separation of the pro-bud a number of form changes occur. The probud elongates along its original axis of growth as expressed by the epicardial axis until it becomes two or three times as long as it is wide. At the same time the inner vesicle invaginates along its longer axis, seen in optical end section in Text-fig. 16C and 17B, and in lateral view in Text-fig. 16D. While this dual change takes place in the probud, the stump of epicardial and epidermal tissue of the parent zooid continues to elongate as a long tubular process, shown in Text-figs. 11B and 16D. At its tip the epidermis and especially the epicardial epithelium is thickened like the part originally constricted off as the probud. A little later this tip of growing tissue is in turn constricted off to form a double-walled sphere similar to the probud in its initial state except that it is relatively minute (Text-fig. 16E).

At the same time the probud attains its maximum length and constrictions appear in it initiated by the epidermis. These may appear simultaneously as two constrictions, as in Text-fig. 16E, or one may form as that shown in Text-fig. 17A, a second appearing somewhat later. In any case there is a definite tendency for the constriction nearest the original proximal end of the probud to appear first. The constrictions deepen until the entire elongated mass of the probud is divided into three more or less spherical buds. Of these the proximal bud has a mass markedly greater than the distal buds.

There are accordingly, as a rule, four buds produced in the tunic of the tadpole larva of *D. bermudensis*, the probud giving rise to one large and two medium-sized buds, the epicardial stump adding a fourth and comparatively minute bud. Of the four, only the largest shows a recognizable extent of morphogenesis at the time of the mature active tadpole. In this species the ultimate fate of the four has not been determined, but in the strictly comparable situation in *D. rosea* (p. 255) only the largest bud eventually develops
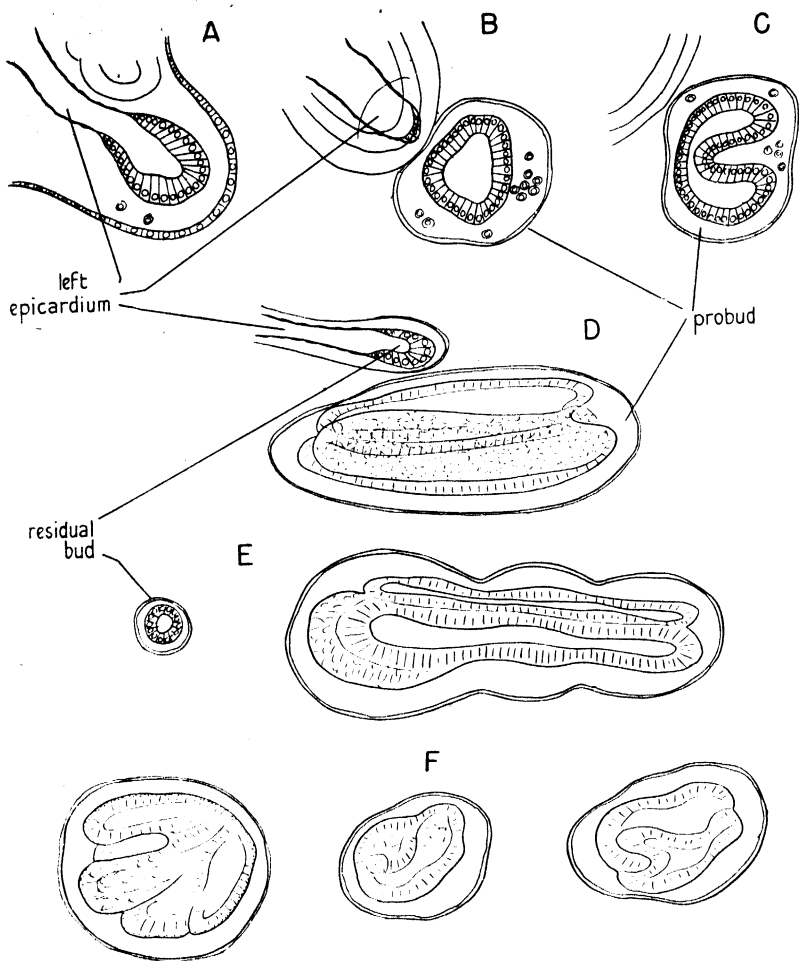
TEXT-FIG. 16. Bud production in tadpole of *D. bermudensis*. A. Initiation of probud as local outgrowth of tip of left epicardium and overlying epidermis. B. Segregation of probud. C. Doubling of epicardial component of probud. D. Elongation of probud and extension of residual epicardial tip. E. Strobilation of the probud and segregation of residual epicardial tip. F. Three definitive buds formed from probud.

to the functional state, the next largest attaining an early but arrested developmental stage, while the remainder disappear.

Probuds are to be seen both in the tadpole larva and in adult colonies. As such they have been reported in the larva and colony of *D. magnilarva* and *D. rosea*. They are equally typical of *D. garstangi* and *D. bermudensis* and the probuds of a mature colony of *D. garstangi* are shown. In each case it is clearly evident that the constriction of the probud into definitive buds is

entirely an epidermal action, the epidermis developing a progressively
deepening constriction that strangulates the epicardial tissues within.

It is equally evident that the constriction appears in such a place that a
sphere is segregated, the size being determined by the short axis of the probud
already formed. In the comparatively long probud of the larva at least three
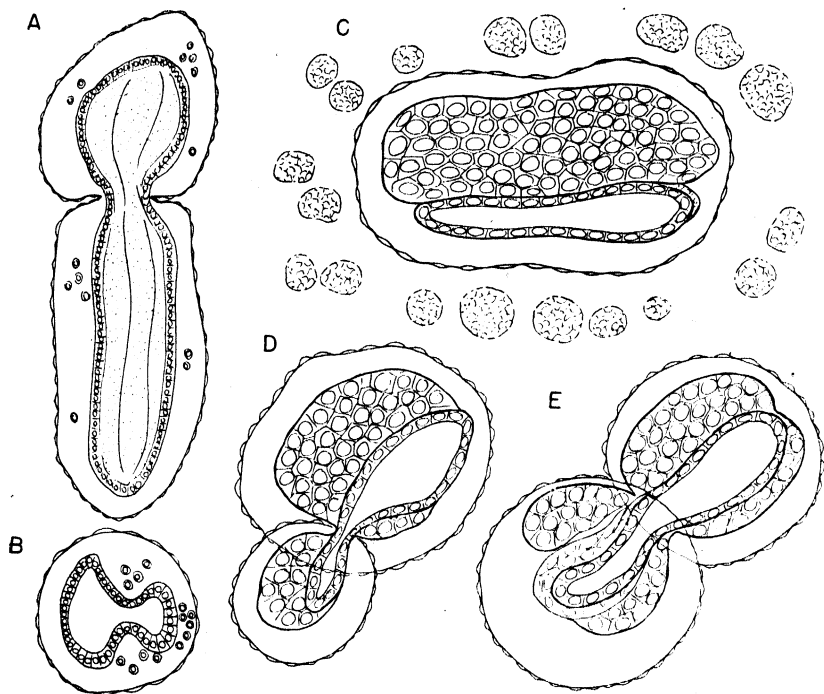


TEXT-FIG. 17. Probuds. A. Probud of tadpole of *D. bermudensis* showing primary con-
striction. B. End view of same to show doubling of epicardial component. C. Probud
of mature blastozooid of *D. garstangi* showing doubled and differentiated epicardial
component. D, E. Similar probuds in process of unequal constriction.

spheres are formed, but in the probuds of mature colonies the long axis rarely
equals twice the diameter, the closest to this condition usually seen being that
of Text-fig. 17C. The earliest stage in the constricting process is discernible as
a slight saddle dividing the probud into approximately equal parts. When a
probud is only slightly longer than it is wide, it is not divided equally but is
constricted so that a maximum-sized sphere is cut off from one end, leaving
a small residual sphere at the other (Text-fig. 17D). Frequently the epidermal
layer of the probud is approximately twice as long as it is wide, but the con-
tained epicardial component has a long axis that is considerably shorter than
this. In such cases the epidermal constriction divides the outer layer
to form epidermal vesicles of equal size, but the internal epicardial tissue,

lying more towards one end than the other, is cut unequally (Text-fig. 17E). The epicardial component accordingly plays a passive role throughout with regard to the process of subdivision.

In Text-fig. 17C the probud is surrounded by numbers of large reserve-laden trophocytes. These tend to accumulate around all young buds and must be actively attracted to these positions. They are characteristic only of buds in established colonies, and have not yet been produced in the larva.

A further comparison may be made between the probuds found in larva and colony. In the larva the epicardial vesicle doubles or invaginates along its main axis as shown in Text-fig. 17B. Even in this case the larger division rapidly loses its lumen and becomes a solid tissue. In those of the colony the same doubling of the epicardial component occurs, but what was the smaller division is now the larger and consists of a compact mass of relatively large cells (Text-fig. 17C, D, E). The division of the epicardial tissue into these two distinct regions is definitely related to the morphogenesis of the definitive buds, and represents primarily a segregation of the reproductive and other mesodermal tissue from the rest. The significant feature here is that a basically important phase of morphogenesis has already commenced at a time when only two of the three dimensions of the organism are in existence. Essentially the same phenomenon occurs in the process of bud formation in *Salpa* and *Pyrosoma*.

It is evident therefore that *Distaplia* buds exhibit two variables, in the gross size of the bud and in the quantity of epicardial tissue. Those of the larva have a size range as a whole considerably smaller than those of the colonies, with only one bud large enough to ensure development. The others appear to be abortive as a result of being of too small an initial size, a phenomenon already recognized in the case of *Stentor* fragments (Lillie, 1896), *Hydra* (Papenfuss, 1934), *Clavelina* buds (Berrill and Cohen, 1936), and *Botryllus* (Berrill, 1941). Of the buds produced in colonies, both large and small divisions of a probud are usually capable of development, though large and small zooids are respectively formed. The relationship between size of equivalent stages and initial bud size is perhaps shown with greater certainty in comparing the development of the larval bud and the average large bud produced in a colony. Such a comparison is shown in Text-fig. 18, A and B being two stages in the development of the larval bud, and C and D exactly equivalent morphological stages of the colony bud. In Text-fig. 18A and C the bud is at its earliest stage of differentiation, very shortly following its segregation from the probud, and in each case consisting of an epidermal vesicle containing a hollow and a compact tissue mass. The hollow structure and the adjacent mass are both derived from the original epicardial sac. In Text-fig. 18B and D is shown an identical morphological stage representing in each case an equivalent degree of gross expansion or growth of the primary stage, yielding a large and small young bud respectively. In both small and large, however, the inner hollow structure has differentiated to form a neural
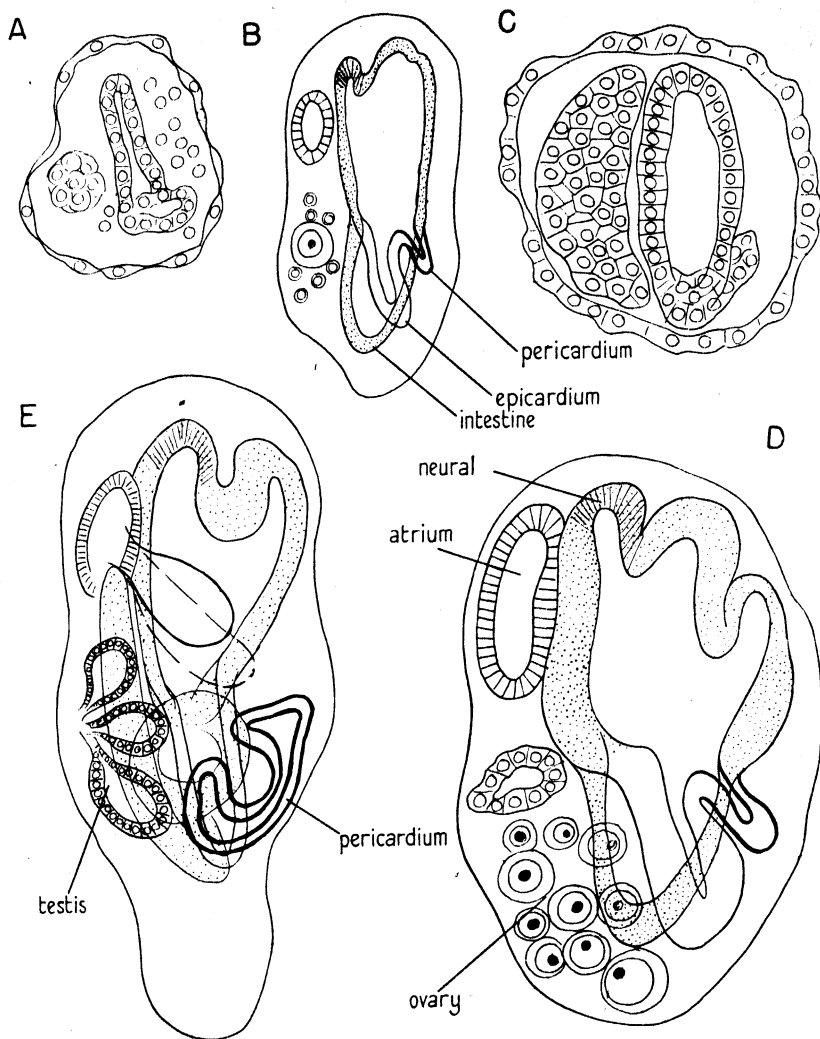
TEXT-FIG. 18. Early morphogenesis of bud of *D. rosea*. A, B. Two stages in development of bud of tadpole. C, D. Equivalent stages in development of relatively larger bud of mature blastozooid. E. Later stage in development of tadpole bud (i.e. first blastozooid generation). N.B. variability in gonad initiation.

evagination dorsally, and ventrally to form three evaginations representing the intestine, the epicardium, and the pericardium. In other words, the size of the primary stage when first constricted from the probud determines the size of subsequent stages up to the time the bud completes its development as such and becomes functional.

A further variable is in the relative mass of the central hollow vesicle and the adjacent compact tissue from which the gonads and some other tissues are derived, a contrast shown in Text-fig. 18A and c. The variable quantity of the presumptive gonad tissue seems to be the cause of the great variability in the nature of the gonads subsequently developed. The buds produced by the
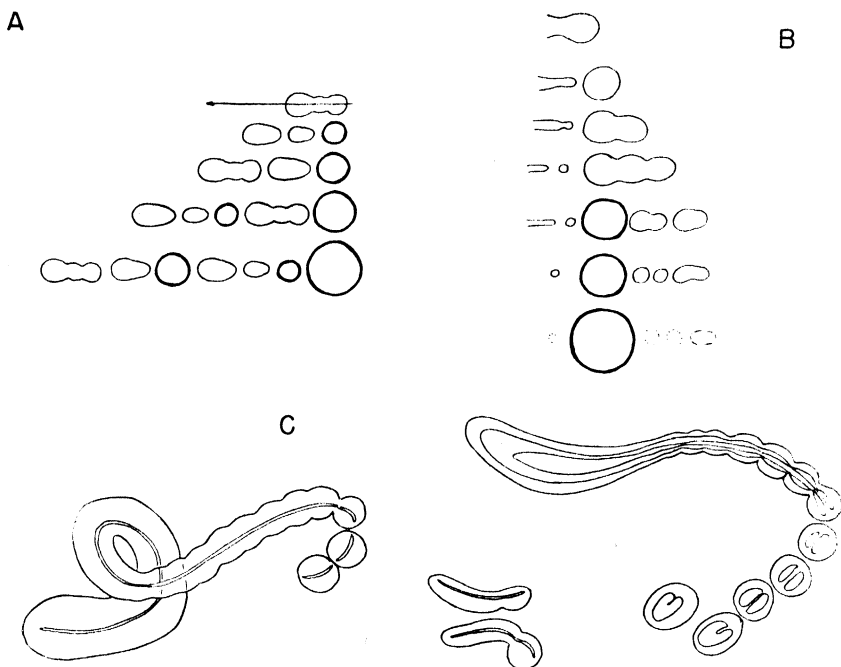


TEXT-FIG. 19. A. Scheme of perpetual bud production by probud proposed by Brien. B. Alternative interpretation (for explanation see text). c. Strobilation of the pro-buds of *Colella pedunculata*, *C. racovitzai*, and *C. cerebriformis*, after Van Beneden and De Selys-Longchamps. (Probuds thin-lined, definitive buds thick-lined in A and B.)

oozooid do not exhibit any discernible sign of gonads of either sex when fully developed, in the case of *D. rosea*, but early stages show rudimentary gonads of either sex though never both together (Text-fig. 18B and E). It has been shown elsewhere (Berrill, 1941) in the case of *Botryllus* that the initial size of the presumptive gonad area determines whether the bud subsequently developed is asexual, immature or mature male, or a mature functional hermaphrodite. The same relationship appears to hold here, and mature ovaries develop only when the presumptive gonad initially segregated is quantitatively adequate. Submaximal masses may well give rise to main components only, as in *D. magnilarva*.

## DISCUSSION

A very full review of the various interpretations of larval budding in *Distaplia* has been given by Brien (1939). It does not seem necessary to repeat this, other than to present his conclusions from this section (p. 113). These are that the very young larva emits a single bud, 'probud' or 'primordial bud' (Della Valle) formed by two concentric vesicles, an external vesicle derived from the medioventral ectoderm of the larva, an internal endoblastic vesicle, derived from the extremity of the left epicardium of the oozooid and between the two vesicles, mesenchymatous elements (Della Valle, Salensky, Julin, Caullery), which continue to divide in the colony (Caullery, Salfi), giving rise progressively to all the ascidiozooids of the colony.

Brien's own investigations are based upon this premiss, that the adult blastozooids have no power to bud and consequently the larval probud must give rise to all buds. With this in mind he interprets the succession of divisions of the probud in a most ingenious way, so that a series of both definitive buds and new probuds are continuously produced; in place of simple growth a single and unique proliferative stolon, successively liberating distal blastozooids, suffers a second constriction more proximal and almost synchronous with the distal blastogenetic constriction. This second proximal constriction should be called the proximal constriction of subdivision because it divides the proliferative stolon into two secondary proliferative stolons which will follow the same destiny as the primary proliferative stolon (Text-fig. 19A).

This concept does bring the budding process of *Distaplia* into line with that of *Colella*, and it does not conflict with the general picture of the probud and the definitive buds as seen in the tadpole larva, and is plausible if the fate of the probud subdivisions is unknown and if no other source of probuds or buds is known.

In the present account, in this connexion, the evidence is that of the several units produced by constrictions of the larval probud, the largest one or two develop into blastozooids and the smaller degenerate and disappear, there being no sign of probuds at the time of dissolution of the oozooid. Further, and of even greater significance, is the discovery that the adult blastozooids give rise to probuds, capable of producing at least two definitive buds, during the last phase of their own dissolution. There is accordingly no need to read a self-perpetuation process into the constrictions of the larval probud.

The activity of the probud is consequently interpreted here in a different and simpler manner. The definitive bud at its inception from the probud is from the hinder end that actually broke contact with the parent tissues. In the sense of origin this should be the proximal end, and the distal end the emergent epidermal-epicardial tip originally protruding from the parental abdomen.

The present account regards the probud as the constricted distal end of the abdominal outgrowth, separated off and continuing to elongate to a considerable extent after separation, and that the elongated probud undergoes further

constrictions to form spheres of sizes varying according to the probud diameter at the time. Only buds exceeding an initial critical size undergo further development, the rest atrophying. The large proximal (in our sense) bud develops, the most distal undergoes some morphogenesis but usually is abortive, while the smaller intervening buds never start (see Text-fig. 19B).

If Brien's reversal of the morphological proximal and distal ends of the probud were valid, the process of budding would be analogous and possibly homologous with that of the Thaliaceans mentioned above. In these forms constrictions occur in series from the distal end of the stolon and the proximal end remains attached to the parent. Actually in *Distaplia* constrictions commence at the opposite end, and the matching is therefore inaccurate. The nature of the stolon or probud from the point of view of origin, however, is significantly similar both with regard to tissues and site.

The relationship between *Distaplia* and *Colella* is another matter. These are genera clearly belonging to the same family. The budding process and morphogenesis of the bud are essentially the same in both. In the colonies of each, probuds in process of constriction are commonly seen, and in *Colella* as in *Distaplia* with no discernible connexion with a parent zooid. Consequently the question which is the original distal and proximal end of the relatively long probuds of *Colella* is debatable, but it is more plausible to consider them the same as in *Distaplia*. The origin of the probuds of *Colella* has been as obscure as that of *Distaplia* colonies and the explanation in the case of *Distaplia* may well be the explanation of both. This is supported to some extent by Herdman's figures of *Colella pedunculata* (1886, Pl. IX, figs. 3, 4, 5, 18), which are remarkably similar to the process illustrated here in Text-fig. 15.

The various species of *Colella* exhibit the same peculiar sexual conditions as *Distaplia* species, though shown in more extreme form. It is unfortunate that this exclusively subantarctic genus is so difficult to obtain, for it merits much more extensive investigation than has yet been possible. *Colella gaimardi*, *C. pulchra*, *C. concreta*, *C. elongata*, and *C. Thomsoni* have hermaphrodite gonads like those of *Distaplia bermudensis*, &c., but *Colella murrayi*, *C. ramulosa*, *C. quoyi*, *C. cerebriformis*, and *C. incerta* are said to be rigorously unisexual. The genus *Holozoa* (*Julinia*, Calman, 1895) is of equal interest and obscurity (cf. Hartmeyer, 1911), from the points of view of budding, sexuality, and life-cycle.

## SUMMARY

The complete developmental cycle, sexual and asexual, is described for several species of the ascidian genus, *Distaplia*.

*Part 1*. The cycle of *D. rosea* is described, from the active larva to the establishment of the first blastozooid generation. Of the four or five buds produced from the larval probud, only one develops, the rest remain abortive. Some larvae are often retained in the colony after the dissolution of the parent zooid and give rise to blastozooids (Text-figs. 1–3).

The general morphology of mature zooids is described for *D. bermudensis*, and their arrangement into typical systems in the colony. Zooids are at first functional males, while the brood-pouch develops later as the first egg in the ovary approaches maturity. The brood-pouch is essentially a loop of the oviduct and does not involve the atrial wall (Text-figs. 4–5).

In the gross development of the bud up to the time it becomes functional, the originally spherical bud elongates relatively along its antero-posterior axis but otherwise does not exhibit differential growth. After the functional state is reached, differential growth of the atrial siphon commences and eventually transforms the small circular siphon into the relatively enormous atrial aperture of the mature zooid (Text-figs. 4 and 6).

Gonads develop as hermaphrodite structures in buds of *D. rosea*, *D. bermudensis*, *D. occidentalis*, and *D. clavata*, but as either ovaries or testes alone in *D. magnilarva* (Text-fig. 7).

*Part 2*. Tadpole larvae of *Distaplia* vary greatly in size according to the species, but not in organization, the cell number of purely larval structures such as adhesive organs and sensory organs being constant (Text-fig. 8).

Cleavage of the egg, in spite of large size and yolk content, is typical of ascidians in general, and shows bilateral patterning comparable with that of *Styela* and *Ciona* (Text-fig. 9).

There is great growth in the development from the egg to the tadpole stage, though the full number of cells of both the notochord and tail muscle is reached at an early stage, about 40 in the case of the notochord (as in all other ascidians) and over 2,000 tail-muscle cells (the largest number known). In the later development of the tadpole larva, the anterior region bearing the adhesive organ and the ampullae becomes progressively constricted off until it remains attached only by a vascular stolon. The probud is separated from the abdomen at a precise stage in the whole development and subdivides before tadpole development is complete (Text-figs. 10–12).

The sensory vesicle contains a typical unicellular otolith and an ocellus consisting of optic pigment cup, layer of retinal cells, and three unicellular lens cells. In the tail the intracellular notochordal vacuoles run together to form a continuous fluid cylinder with the chordal cells forming a limiting sheath. Myofibrillae are continuous from cell to cell in the lateral interfaces of the ectoplasmic regions (Text-fig. 13).

*Part 3*. Contrary to all previous reports, buds are produced by adult zooids and are not confined to the tadpole larva, but they are produced at a different phase of the life-cycle of the individual zooid. After sexual reproduction is complete, the zooids atrophy, their tissues autolysing and becoming phagocytosed in part. All of the zooid degenerates with the exception of the epicardium and surrounding epidermis. This structure actually undergoes extensive elongation, at the close of which the posterior end of the pericardium and enclosing epidermis constricts off as one of two probuds (Text-figs. 14–15).

This is essentially the same process, apart from the stage with which it is associated, as occurs in the tadpole larva. In both cases the probud elongates

and constricts into two or more definitive buds (Text-figs. 16–17), the smallest of which fail to develop.

The formation of hermaphrodite or unisexual gonads depends primarily on the mass of the presumptive gonad tissue segregated at a very early critical period in bud morphogenesis (Text-fig. 18).

The process of bud formation is compared with that of the allied genus *Colella*, and an interpretation very different from that of Brien is presented (Text-fig. 20).

## REFERENCES

BANCROFT, F. W., 1899. 'Ovogenesis in *Distaplia* occidentalis Ritter (M.S.) with remarks on other species.' Bull. Mus. Comp. Zool. Harvard, **35**, 59.

BENEDEN, E. VAN, and DE SELYS-LONGCHAMPS, M., 1913. *Résultats du Voyage du S. Y. Belgica. Zoologie: Tuniciers Caudichordata (Ascidiacés et Thaliacés)*.

BERRILL, N. J., 1935. 'Cell division and differentiation in asexual and sexual development.' J. Morph., **57**, 353.

—— 1941. 'Spatial and temporal growth patterns in colonial organisms.' Growth, V. (Symposium Supplement), p. 89.

—— and COHEN, A., 1936. 'Regeneration in Clavelina.' J. exp. Biol., **13**, 352.

BRIEN, P., 1939. 'Contribution à l'étude du bourgeonnement et de l'organogenèse du blasto-zoïde des Distomidae (=Polycytoridae) [Distaplia magnilarva = Holozoa magnilarva].' Ann. Soc. zool. Belg. **70**, 102.

CALMAN, W. T., 1895. 'On *Julinia*, a new genus of compound ascidians from the Antarctic Ocean.' Quart. J. micr. Sci., **37**, 1.

CAULLERY, M., 1895. 'Contributions à l'étude des ascidies composées.' Bull. Sci. Fr. Belg., **27**, 1.

—— 1909. 'Recherches sur les Synascidies du genre *Colella* et considérations sur la famille Distomidae.' Bull. Sci. Fr. Belg., **42**, 1.

CONKLIN, E. G., 1905. 'The organization and cell lineage of the ascidian egg.' Journ. Acad. Nat. Sci. Philadelphia, **13**, 1.

DAUMEZON, G., 1909. 'Contributions à l'étude des Synascidies du Golfe de Marseille.' Bull. Sci. Fr. Belg., **42**, 269.

DAWIDOFF, M., 1889. 'Untersuchungen zur Entwicklungsgeschichte der *Distaplia magnilarva*, Della Valle, einer zusammengesetzten Ascidien.' Mt. Stat. Zool. Neapel, **9**, 113, 533.

DELLA VALLE, A., 1882. '*Distaplia*, nouveau genre de Synascidies.' Arch. Ital. Biol., **1**, 193.

HARTMEYER, R., 1911. 'Die Ascidien der deutschen Südpolar-Expedition, 1901–1903.' Deutsche Südpolar Exped., **12**, 402.

HERDMAN, W. A., 1886. 'Report on the Tunicata collected during the voyage of H.M.S. "Challenger" during the years 1873–76.' Zool. Challenger Exped., **14**.

JULIN, C. L., 1896. 'Recherches sur la blastogenèse chez *Distaplia magnilarva* et *D. rosea*.' Compt. rend. Congr. Internat. Zool., **3**, 507.

LILLIE, F. R., 1896. 'On the smallest parts of *Stentor* capable of regeneration.' J. Morph., **12**, 239.

PAPENFUSS, E. J., 1934. 'Reunion of pieces in *Hydra*, with special reference to the role of the three layers, and to the fate of differentiated parts.' Biol. Bull., **67**, 223.

SALENSKY, W., 1893. 'Morphologische Studien an Tunicaten *Distaplia magnilarva*.' Morph. Jahrb., **20**, 48.

SALFI, M., 1925. 'La fissazione della larva e la genesi della colonia in *Holozoa magnilarva* (Della Valle).' Boll. Soc. nat. Napoli, **37**, 224.

—— 1933. 'Le recenti ricerche sui fenomini di reproduzione osessuata dei Tunicata.' Arch. Zool. Ital., **19**, 121.

SCOTT, F. M., 1946. 'The developmental history of *Amaroucium constellatum*. II.' Biol. Bull., **91**, 66.

ULJANIN, B. N., 1885. 'Bemerkungen über die Synascidiengattung *Distaplia* D.V.' Zool. Anz., **8**, 40.

# An Egg-waxing Organ in Ticks

BY

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**With one Plate**

## CONTENTS

## INTRODUCTION

MOST general accounts of the natural history of ticks include a brief description of the remarkable oviposition movements encountered in this group. As is well known, each egg, on leaving the vagina of the egg-laying female, is received for a few moments by a glandular organ—the organ of Géné—which is everted shortly before oviposition. The close connexion of this structure with oviposition is emphasized by the fact that it is absent

both from the male and from the immature stages. Its presence, however, is common to both families of ticks: in the Ixodidae it is everted from the space between the basis capituli and the scutum; in the Argasidae it appears from the camerostomal depression. No other groups of Acarina are known to possess the organ.

Nevertheless, despite the fact that this interesting structure was first described just one century ago, our knowledge of its morphology, and more particularly of its function, remains meagre. In one of the earliest accounts of oviposition in ticks, Géné (1848) had noted that when the organ was pricked with a needle the eggs deposited near the vagina soon shrivelled. But he believed that the organ itself served as a receptaculum seminis. In a later experiment by Bertkau (1881), eggs were prevented from coming into contact with Géné's organ by touching the latter with a glass rod and so causing it to retract at the critical moment. Such eggs, he found, subsequently shrivelled much more rapidly than eggs laid in the normal manner. These descriptions both refer to unidentified ticks, possibly *Ixodes ricinus*.

More recent work has been entirely descriptive. Lounsbury (1900) described how in *Amblyomma hebraeum* the organ 'gradually unfolds its glistening arms . . . grasps the egg and apparently envelops it in slime', and Wheler (1906) in a brief account of oviposition in *I. ricinus* also referred to the 'glutinous surface' of the organ. Nuttall (1908) mentioned that in *Haemophysalis punctata* the two vesicles of Géné's organ contain a hyaline secretion but did not suggest a function. The internal glandular part of the organ has been variously referred to by other authors as the 'ovipositing gland', the 'cephalic gland' (Christophers, 1906), or as the 'subscutal gland' (Samson, 1909).

These descriptions suggested to us that the function of Géné's organ is to provide the eggs with a waterproof covering—a function proposed indeed by Bertkau (loc. cit.) but never confirmed. Our studies, which are described in this paper, have shown that a waterproofing agent is undoubtedly transferred from Géné's organ to the egg and that the agent in question is a wax. Now, although it is known that the integuments of many insects (Wigglesworth, 1945; Beament, 1945) and ticks (Lees, 1947) owe their waterproof properties to a thin superficial layer of wax which is secreted through the pore canals, the mode of transport of the wax through the cuticle remains obscure. The occurrence of a waterproofing organ, from which small quantities of material can be isolated, therefore provides a very favourable opportunity for examining the wax precursors. At the same time, however, a more detailed investigation of the structure of Géné's organ has been needed in order to determine the precise site of secretion of the waterproofing agent.

Other questions have also been raised. The unusual nature of the waterproofing process called for a more extended examination of the properties of the wax in relation to those of the substrate, namely, the egg-shell. And this in turn has led us to follow the development of the egg-membranes and to examine some of their chemical and other properties.

Most of our observations have been made on two species of ticks, *Ixodes ricinus* L. and *Ornithodorus moubata* Murray, which were selected as representative of the Ixodidae and Argasidae respectively. All ticks lay their eggs in large clusters. An engorged female *Ixodes* deposits a single cluster of some 2,000 eggs before she is spent. *O. moubata* lays, between successive blood meals, as many as 6 egg batches, each containing about 100 eggs. The small egg of *Ixodes* is elliptical in shape, the much larger egg of *Ornithodorus* ovoid or sub-spherical.

## OVIPOSITION MOVEMENTS

Oviposition has been observed most closely in *I. ricinus*. Engorged female ticks were laid, ventral side uppermost, on the floor of a moist chamber and were secured in this position by means of plasticine bands; they were then left in darkness under a binocular microscope until egg-laying began. Mechanical disturbance or the access of light causes the suspension of oviposition, but usually a few eggs are laid in the light before the rhythm is interrupted. The sequence of events is illustrated in Text-fig. 1.

In the normal position of rest between the delivery of successive eggs, Géné's organ is not visible, the capitulum is directed anteriorly, and the vagina is slit-like (Text-fig. 1A). The delivery of an egg is heralded by the downward movement of the capitulum, the tip of which describes a vertical arc and finally comes to lie closely applied to the ventral body-wall just in front of the vagina (Text-fig. 1B). Géné's organ is then everted from between the dorsal posterior borders of the capitulum and the anterior margin of the scutum. Owing to the movement of the capitulum it protrudes ventrally and comes to lie over the hypostome and palps. The organ itself, when fully extended, is seen to consist of a balloon-like base which is produced on each side into two short horns which contain a translucent fluid (Text-fig. 1C). The whole organ is invested with an exceedingly delicate glistening cuticle, capable of a high degree of folding.

When oviposition is about to take place the hypostome is tucked firmly against the ventral body-wall, the palps are splayed out, and Géné's organ is inflated to its maximal extent. At the same time the inner lining of the vagina begins to unroll like the finger of a glove (Text-fig. 1D). The prolapsed vagina or 'ovipositor' forms a tube which, when fully extended, nearly touches Géné's organ and is a most effective instrument for delivering the egg. After depositing the egg between the horns, the 'ovipositor' is quickly retracted. Géné's organ with the egg attached is then partially deflated and inflated several times and the palps are often worked sideways in an active manner. Finally, the organ is retracted completely within the body and the egg is left adhering to the dorsal surface of the hypostome (Text-fig. 1E, F). The cycle is completed when the hypostome swings into the dorsal position again, carrying the egg with it (Text-fig. 1G). Because of these events the eggs tend to accumulate on the dorsum of the tick and not on the ventral surface, and as
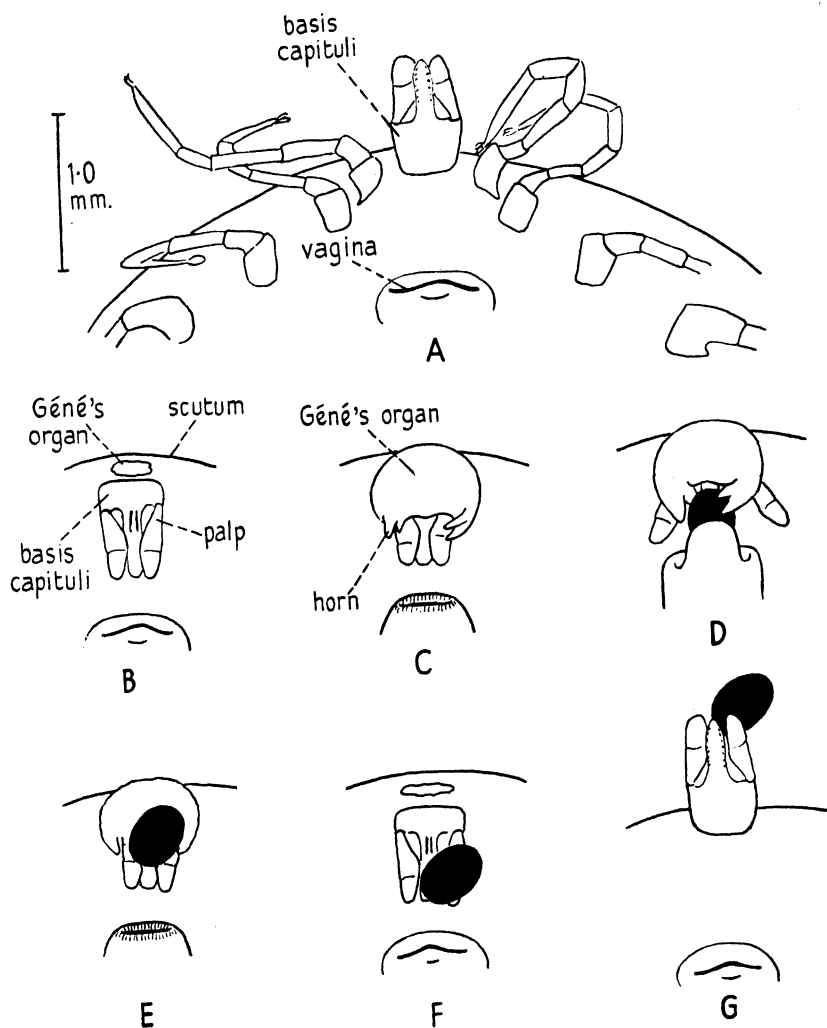
TEXT-FIG. 1. Oviposition movements in *Ixodes ricinus*.

the egg-laying female rarely moves after the onset of oviposition the eggs are deposited in a dense cluster.

The rate of egg production under favourable conditions is not easy to determine, as after any disturbance laying is not resumed for some hours. One female tick laid as many as 246 eggs during 48 hours, that is, at the rate of 1 egg every 12 minutes. According to Wheler (1906), however, the sheep tick may sometimes lay eggs at the rate of 1 every 3 minutes.

· The movement of the palps and of Géné's organ itself may sometimes have the effect of rotating the egg and so promoting increased contact with the shell. However, careful observation has convinced us that as a general rule only a small area of the shell—certainly less than half the total surface—establishes contact with Géné's organ. At this time the egg usually lies between the horns which may embrace it as the organ is retracted. Nevertheless contact with the horns is not essential, for eggs which chance to be deposited on the dorsal aspect of the organ, where the horns cannot reach them, undergo normal development subsequently. Once oviposition is under way the cuticle of Géné's organ soon acquires a greasy glistening appearance and the film of grease gradually extends to the legs and the cuticle near the vagina. However, spreading of the grease to the egg-shell cannot be observed under the microscope.

The oviposition movements in *Ornithodorus moubata* have not been followed in detail as this tick is even more sensitive to disturbance, but the appearance of females which have been interrupted at different stages of egg-laying suggests that the process is the same in all essentials. Co-ordinated movements involving the eversion of Géné's organ, the prolapse of the vaginal lining, and the rotation of the hypostome probably take place as in *Ixodes*. Since the hypostome is shorter, however, and the eggs less sticky, the latter are not carried dorsalwards but instead accumulate in a heap beneath the anterior region.

### VISUAL DEMONSTRATION OF THE SECRETION OF WAX BY GÉNÉ'S ORGAN

The role of Géné's organ in secreting a wax is most simply demonstrated in *O. moubata*. In this species the organ can be caused to evert if the body of the tick is compressed between two microscope slides so that only the proboscis and camerostome project freely (Pl. I, fig. 1). After the slides have been clipped together with Cornets forceps the organ can be held everted for further detailed examination. A full description will be deferred until a later section.

In the normal egg-laying female the surface of Géné's organ is always devoid of large accumulations of wax. If, however, engorged ticks are kept for 3 or 4 weeks at 15° C.—a temperature just too low for oviposition—large amorphous deposits of wax appear on the surface of the cuticle, usually around the base of the horns. These can easily be collected on a needle after everting the organ by pressure. Wax accretions on three organs are shown in Text-fig. 2; not all individuals secrete wax in such quantity.

That Géné's organ in *Ixodes* is also concerned in secreting a wax can be shown by everting the organ and touching it against a clean glass slide. The demonstration is less striking than in *Ornithodorus*, however, for in this species the wax fails to accumulate in any quantity owing to its greater mobility (see p. 302).

### The Fate of Eggs laid out of Contact with Géné's Organ

The function of Géné's organ in waterproofing the eggs has been confirmed by occluding the opening through which the organ is everted and following the subsequent fate of the eggs when exposed to different humidities. In *Ornithodorus*, eversion was prevented by covering the camerostomal fold and proboscis with cellulose paint. This somewhat delays the onset of oviposition but afterwards egg-laying continues at the normal rate. The procedure adopted was to allow the same ticks, isolated singly in specimen tubes, to lay successive small batches of eggs with the opening free, then occluded, then



TEXT-FIG. 2. Natural accumulations of wax on Géné's organ in *Ornithodorus*.

finally free again. The laying ticks were exposed to a humidity of 70 per cent. R.H. in an incubator at 25° C. where the eggs remained undisturbed for 14 days. At this temperature all normal eggs hatch within 12 days.

Some of the results obtained are given in Table 1. As a general rule (e.g. ticks, nos. 1–3) very nearly all the eggs laid with Géné's organ free hatch normally, while those laid without the intervention of the organ are all completely shrivelled and hard. Sometimes, however, a tick which at first lays normal eggs continues to lay shrivelling eggs after Géné's organ has been freed (e.g. no. 4). This may be ascribed to the disturbing influence of covering and uncovering the camerostome which probably upsets the synchronous eversion of the organ. The eggs failing to touch Géné's organ will therefore remain unwaterproofed. A few shrivelling eggs are sometimes laid among or preceding an otherwise normal batch (e.g. no. 1). No doubt this is also caused by an occasional faulty eversion of Géné's organ. The same phenomenon is seen when egg-laying ticks are kept in crowded cultures where mutual disturbance is often sufficient to lead to the production of large numbers of unwaterproofed eggs.

Unless contact with Géné's organ influences the viability of the egg in other ways, a proportion of the eggs deprived of contact would be expected to hatch if the atmosphere were kept sufficiently moist. A further group of egg-laying females with Géné's organ obstructed was therefore set aside in saturated air and was examined only when hatching should have been complete. Six females laid a total of 386 eggs of which 23 hatched, the remainder, even in

the damp atmosphere, gradually collapsing and darkening. One hundred and eighty-four eggs, which were laid by 3 ticks, yielded no nymphs. One female, on the other hand, laid 28 eggs of which as many as 14 hatched. Normal egg-masses kept in a damp atmosphere remain free from moulds almost indefinitely. It was noteworthy that eggs laid without the intervention of Géné's organ rapidly acquired a thick felt of hyphae growing on the surface of the egg-shell. This is a further result of the absence of wax from the shell.

Parallel observations have been made on *Ixodes ricinus*, with generally similar results (Table 1). Egg-laying females were secured on their backs with

TABLE 1. *The hatching of successive egg-batches laid with or without the intervention of Géné's organ*

The eggs of *O. moubata* and of *I. ricinus* were exposed during incubation to humidities of 70 and 100 per cent. R.H. respectively

| Species | Tick no. | Géné's organ uncovered | | | Géné's organ blocked | | | Géné's organ again uncovered | | |
| | | No. of eggs | | | No. of eggs | | | No. of eggs | | |
| | | Laid | Hatched | Shrivelled | Laid | Hatched | Shrivelled | Laid | Hatched | Shrivelled |
| Ornitho-dorus moubata | 1 | 38 | 36 | 2 | 64 | 0 | 64 | 49 | 49 | 0 |
| | 2 | 22 | 22 | 0 | 47 | 0 | 47 | 53 | 46 | 7 |
| | 3 | 36 | 36 | 0 | 25 | 0 | 25 | 82 | 45 | 37 |
| | 4 | 22 | 22 | 0 | 70 | 0 | 70 | 47 | 0 | 47 |
| Ixodes ricinus | 5 | 998 | 984 | 14 | 308 | 20 | 288 | 579 | 566 | 13 |
| | 6 | 859 | 822 | 37 | 255 | 0 | 255 | 604 | 486 | 118 |
| | 7 | 218 | 208 | 10 | 247 | 2 | 245 | 555 | 213 | 342 |
| | 8 | 171 | 151 | 20 | 154 | 10 | 144 | 216 | 73 | 143 |

the hypostome firmly embedded in plasticine. As this eliminates the hypostomal movements, as well as preventing the eversion of Géné's organ, the eggs collect in a pile round the opening of the vagina. Normal egg batches of this species are far more susceptible to desiccation than are those of *Ornithodorus* (p. 301) and for complete hatching must be exposed continuously to saturated air. The results of observations on the hatching of small egg-clusters, laid consecutively, with or without the aid of Géné's organ, are summarized in Table 1. Again, as the first column shows, there is always a small number of shrivelling eggs in every normal egg-cluster, probably resulting from the failure of the organ to evert properly. With the organ blocked the great majority of the eggs, even in the saturated atmosphere, gradually collapse and darken and very few hatch. After removal of the block greater numbers of eggs hatch, although in many individuals an abnormally high proportion of the eggs prove to be non-viable. As in *Ornithodorus*, therefore, the interference with Géné's organ appears to hinder its proper functioning, even when the obstruction has been completely removed.

DEMONSTRATION OF THE WATERPROOFING LAYER ON THE EGG

If, as has been suggested, a waxy covering is applied to the outside of the egg by Géné's organ, wax solvents and detergents should be capable of

attacking this superficial layer and therefore of exerting a pronounced effect on transpiration through the shell. As Table 2 shows, this expectation is fulfilled.

TABLE 2. *The effect of chloroform and Co9993 on the water loss from small egg-batches (average weight 26 mg.) laid during the previous 24 hours*

| Species | Treatment | Per cent. loss of weight during 1 hr. in dry air at 25° C. |
|---|---|---|
| Ornithodorus moubata | None | 0·7 |
| | Extracted in chloroform at 15° C. for 1 minute | 45·0 |
| | Smeared with Co9993 | 24·0 |
| Ixodes ricinus | None | 1·2 |
| | Extracted in chloroform at 15° C. for 1 minute | 51·0 |
| | Smeared with Co9993 | 15·7 |

Water loss from newly laid egg-masses is greatly increased if they are extracted with cold chloroform or smeared with the wax emulsifier Co9993 (see Wigglesworth, 1945). The effect of chloroform is particularly dramatic on the relatively small eggs of *Ixodes*. Of the normal waterproofed eggs about 50 per cent. shrivel during 24 hours in dry air at 25° C. After washing in chloroform for 30 seconds all the eggs shrivel and dry out completely within 5 minutes. As a result of the removal of the wax, the eggs no longer stick together and tend to roll about freely.

That Géné's organ is concerned with the deposition of the wax layer can be shown by extracting, with chloroform, egg batches which have been laid with or without the intervention of the organ; the relative effect of this treatment on water loss can then be compared. Results with *Ornithodorus moubata* and *Ixodes ricinus* are set out in Table 3. The number of eggs that are shrivelled after a given interval of time can be used as a convenient index of water loss. As it has been found that the permeability of *Ornithodorus* eggs, when devoid of the wax layer, may differ conspicuously from one batch to another, comparisons have always been made using eggs deposited by the same tick.

Normal eggs of *Ornithodorus* never show the slightest shrivelling after 24 hours in dry air at 25° C., while those washed with chloroform are all shrivelled, and some are dry, after only 30 minutes (Table 3). Eggs laid in moist air without the intervention of Géné's organ also shrivel rapidly in dry air; and the rate of shrivelling is not increased after chloroform treatment. Extraction with chloroform also makes little difference to the rate of water loss from eggs dissected from the uterus. Thus it is clear that in this species, the waterproofing material on the outside of the egg is derived solely from Géné's organ.

This is not the case with *Ixodes*, however. Egg-masses deposited with

Géné's organ covered are certainly more permeable than those laid with the assistance of the organ. Thus after 4 hours in dry air at 25° C. none of the eggs in a normal batch of 50 were shrivelled and only a few had begun to dimple, whereas of the eggs laid by the same tick without the organ about half were completely shrivelled (Table 3). Nevertheless, the permeability of the eggs laid without Géné's organ is greatly increased by washing in chloroform; shrivelling is then as rapid as it is after extraction of normal eggs. These results indicate that the egg, before establishing contact with Géné's organ, must already have acquired a covering of some chloroform-soluble material capable of reducing the permeability of the shell. Eggs dissected from the oviduct, on the other hand, are highly permeable; and the permeability is not noticeably increased by chloroform (Table 3).

TABLE 3. *The effect of extracting eggs from various sources with chloroform at 15° C. for 1 minute*

The rate of shrivelling after treatment was observed in dry air at 25° C.
All laid eggs were from batches less than 24 hours old

| Species | How laid | Treatment | No. of eggs | No. of eggs shrivelled after | | | |
|---|---|---|---|---|---|---|---|
| | | | | *5 min.* | *30 min.* | *4 hrs.* | *24 hrs.* |
| Orbitho-dorus moubata | With Géné's organ | None | 20 | 0 | 0 | 0 | 0 |
| | | Chloroform | 20 | 14 | 20 | .. | .. |
| | Without Géné's organ | None | 20 | 8 | 20 | .. | .. |
| | | Chloroform | 20 | 9 | 20 | .. | .. |
| | Eggs from uterus | None | 20 | 16 | 20 | .. | .. |
| | | Chloroform | 20 | 15 | 20 | .. | .. |
| Ixodes ricinus | With Géné's organ | None | 50 | 0 | 0 | 0 | 23 |
| | | Chloroform | 50 | 50 | .. | .. | .. |
| | Without Géné's organ | None | 50 | 0 | 5 | 24 | 50 |
| | | Chloroform | 50 | 50 | .. | .. | .. |
| | Eggs from oviduct | None | 50 | 50 | .. | .. | .. |
| | | Chloroform | 50 | 50 | .. | .. | .. |

It follows that the egg of *Ixodes* is waterproofed in two stages: the egg receives first an external covering of waterproofing material during its passage down the common oviduct and vagina, and then acquires an additional coating from Géné's organ. Further evidence, which is presented below, suggests that both waterproofing agents are waxes with very similar, if not identical, properties. This two-stage application of lipoid can easily be detected if egg-masses laid by the same tick, with and without the intervention of Géné's organ, are compared under the binocular. It is then obvious that although both are visibly greasy, the former are provided with more liberal quantities of wax. The eggs from the oviduct have, in contrast, a smooth, highly polished appearance with no trace of grease.

The greater permeability of eggs that are prevented from touching Géné's organ is due principally to the fact that the first wax layer is always incomplete.

Since the egg-shell contains reducing substances (p. 323), this can be demonstrated by immersing the eggs in 5 per cent. ammoniacal silver nitrate. The shell and yolk of normal eggs always remain completely unstained by this treatment, whereas eggs that have failed to touch the organ invariably show some staining of the shell but little blackening of the yolk. After washing in chloroform there is widespread staining of the shell and very rapid blackening of the yolk as the stain penetrates into the interior of the egg. Whether the thickness of the wax layer also influences the permeability has not been determined.

The eggs laid by different females with Géné's organ out of action vary greatly in permeability. The following is an example. The eggs deposited by 2 ticks on 3 consecutive days were collected and exposed separately to dry air at 25° C. for 4 hours. The egg batches of the first yielded 10, 5, and 2 per cent. of shrivelling eggs, those of the second 35, 44, and 49 per cent. respectively. Individual differences in the coverage of the eggs with wax are probably the cause of this variability.

The presence of the first coating of wax, conferring on the egg a certain degree of impermeability, may account for statements in the literature to the effect that Géné's organ is unnecessary for normal oviposition. Smith (1945) records an example of a specimen of *I. dentatus* which came away from the host with so large a piece of skin still attached to the hypostome that the eversion of Géné's organ was prevented. Yet the eggs deposited by this female appeared normal. With *I. ricinus*, however, we have shown that the first wax covering is insufficient, even in damp air, to prevent the collapse of most of the eggs over the long period required for normal development.

### The Effect of Temperature on Water Loss from the Egg

Previous work with insects (Ramsay, 1935; Wigglesworth, 1945; Beament, 1945), with ticks (Lees, 1947), and with an insect egg (Beament, 1946*b*) has shown that the waxes which are responsible for the impermeability of the cuticle or egg-shell undergo transitional changes at a certain 'critical temperature' and permit water to pass more readily. An evaporation curve exhibiting a pronounced break at a certain temperature can therefore provide confirmatory evidence that a wax layer is present. But in addition it has been found that the critical temperature bears a close relation to the other physical properties of the waxes, which in fact show great variability in different species. Among ticks, species from dry environments, such as the Argasidae, have low rates of transpiration and high critical temperatures; whereas the reverse is true of species from damper environments, like the majority of the Ixodidae.

The effect of temperature on water loss has now been investigated in a representative series of tick eggs. Owing to the small size of the egg it is impracticable to estimate water loss in relation to surface area; for the present purpose of determining the approximate critical temperature it has sufficed to plot loss of weight against temperature. The usual experimental procedure adopted was to expose small clusters of eggs taken from a single egg-mass (weighing about 20 mg. and containing up to 200 eggs) to dry air at different

temperatures for periods of 30 minutes. The eggs were contained in a small gauze basket which was suspended over phosphorus pentoxide in a conical flask immersed up to the neck in a water bath. Owing to the rapid desiccation above the critical temperature, fresh lots of eggs from the main egg-cluster were often used to obtain separate points on the curve.
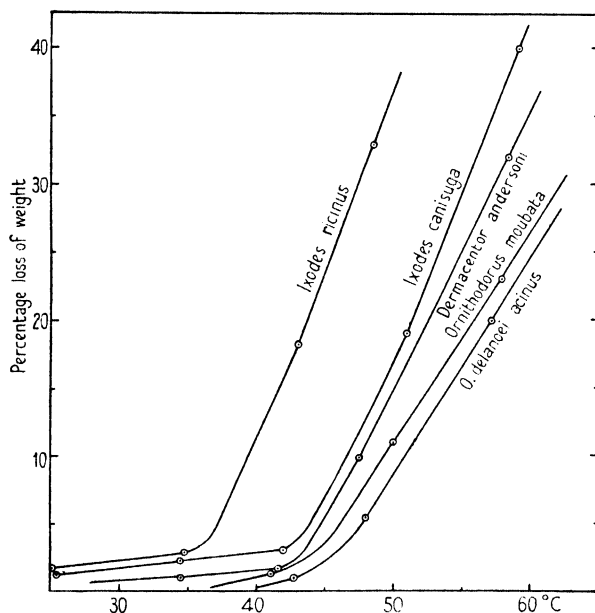


TEXT-FIG. 3. The effect of temperature on the evaporation of water from the eggs of different species of ticks.

The evaporation curves for the eggs of five species are shown in Text-fig. 3. The rate of water loss from egg-masses of comparable weight after exposure to dry air at 25° C. for 24 hours and the approximate critical temperature of the eggs are recorded in Table 4. We also include for comparison the critical temperatures previously obtained for the cuticle of the parent species (Lees, 1947). It should be borne in mind, however, that the values for the cuticle were read off from curves relating transpiration and surface area.

The results bring out several points. First, the different species can be arranged in a graded series according to the resistance of the eggs to desiccation at 25° C. When this is done, the sequence corresponds closely with a similar series based on the order of resistance of the adult tick itself (Lees, 1947). In other words, susceptible species, such as *Ixodes ricinus*, also lay susceptible eggs; more resistant species, like *Ornithodorus*, lay comparatively resistant eggs, and so on. Secondly, the critical temperature is related to the rate of water loss below the critical temperature (e.g. at 25° C.). Thirdly, the

critical temperature of the egg in Ixodidae is nearly identical with that of the cuticle (the difference of 3° C. in the case of *I. ricinus* is of doubtful significance); whereas the critical temperature of the egg in Argasidae is much lower than that of the cuticle. In *O. moubata*, for example, we obtained values of 45° C. and 62° C. for the egg and cuticle respectively. The egg critical temperature is only 1° C. above that of the most resistant Ixodid, *Hyalomma savignyi*.

TABLE 4. *Water loss from small batches of eggs (weighing about 20 mg.)
and their approximate critical temperatures*

The critical temperature of the female ticks are included for comparison

| Family | Species | Per cent. loss of weight in dry air during 24 hrs. at 25° C. | Critical temperature, ° C. | |
|--------|---------|------------------------------------------------------------|------|---------|
| | | | Egg | Cuticle |
| Ixodidae | *Ixodes ricinus* | 27·2 | 35 | 32 |
| | *I. canisuga* | 15·6 | 42·5 | 42 |
| | *Dermacentor andersoni* | 9·0 | 43 | 44 |
| | *Hyalomma savignyi* | 8·0 | 44 | 45 |
| Argasidae | *Ornithodorus moubata* | 7·9 | 45 | 62 |
| | *Ornithodorus delanoei acinus* | 2·5 | 46 | 67 |

Besides differing in their transitional temperatures, the egg waxes in this range of species probably exhibit parallel differences in their other physical properties. We have already recorded that in *I. ricinus* the lipoid secreted by Géné's organ is a labile grease, whereas in *O. moubata* it is a viscous wax. Species with intermediate critical temperatures no doubt possess egg waxes with intermediate physical properties. A difference in 'stickiness' can indeed be easily appreciated merely by handling the egg-masses, for the eggs adhere to one another solely by virtue of their waxy covering. The egg-masses of *I. ricinus*, for example, stick together most tenaciously; those of *H. savignyi* appear much less greasy and fall apart quite readily; while the eggs of *O. moubata* and *O. delanoei* show only the slightest mutual coherence.

We have already pointed out that in the *Ornithodorus* egg the entire waterproof covering is supplied by Géné's organ, whereas the *Ixodes* egg is partly waterproofed before leaving the vagina. Confirmation is provided by the effect of temperature on the water loss from egg-masses of the two species laid with and without the intervention of Géné's organ. Eggs of *Ornithodorus* laid in a damp atmosphere with the organ covered lose water rapidly at all temperatures when exposed to dry air (Text-fig. 4). *Ixodes* egg-masses, laid without Géné's organ, although distinctly more permeable than eggs touched by the organ, still show a definite break in the evaporation curve at about 31° C. (This type of curve may be compared with those obtained by Wigglesworth (1945) for soil-inhabiting larvae of *Tipula* and *Hepialus*. Although the cuticular wax layer of these insects is more or less severely scratched by soil particles, a distinct break in the evaporation curves may still be detected.)

Normal eggs laid by the same ticks had a critical temperature of about 34° C. (Text-fig. 4). This is additional evidence that the partial waterproofing effect is also due to the presence of a wax layer. Probably the wax is similar in nature to that secreted by Géné's organ, although smaller in quantity.
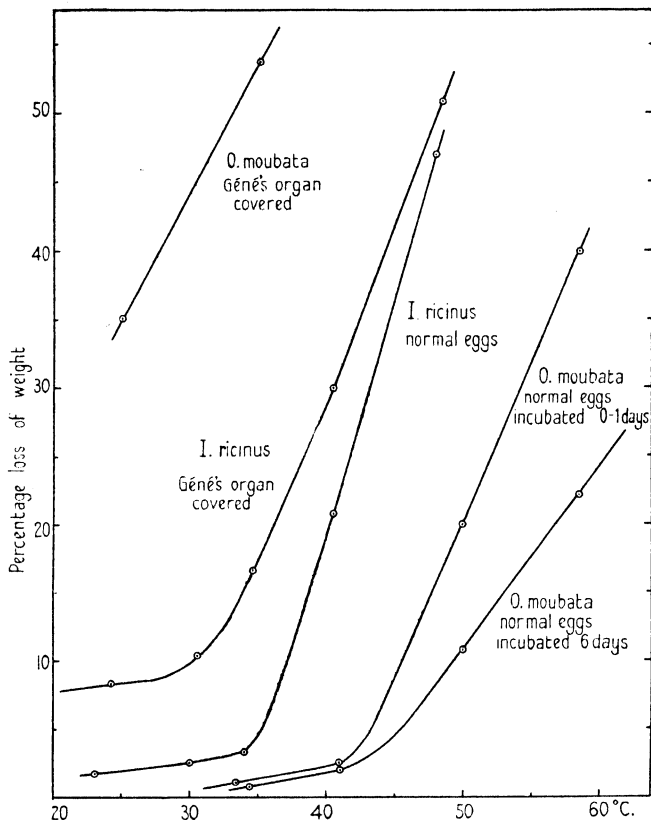


TEXT-FIG. 4. The effect of temperature on the evaporation of water from egg-masses laid with and without the intervention of Géné's organ.

Text-fig. 4 also shows the evaporation curves of *Ornithodorus* eggs 0–1 days after laying and after incubation for 6 days at 25° C. Eggs of both ages have a critical temperature of about 44° C. But the older eggs lose water at a lower rate, particularly at temperatures above 44° C. Some possible explanations of this effect are discussed later (p. 324).

THE MORPHOLOGY OF GÉNÉ'S ORGAN IN *ORNITHODORUS MOUBATA*

The external appearance of Géné's organ in *Ixodes* has already been described briefly. Because of the greater ease of everting the organ in

*Ornithodorus*, we have chosen this species for more detailed study. Much of the internal structure is visible through the transparent cuticle.

The everted organ consists of a broadly sessile stalk surmounted by two large crescentic horns (not four as in *Ixodes*). When it is fully inflated the stalk is completely occupied by the white tissues of the gland (Text-fig. 5 A). The horns are particularly conspicuous as they are usually filled with a clear refractile liquid—evidently the secretion of the gland—which extends also round the margin of the organ at the base of the horns and occupies the space
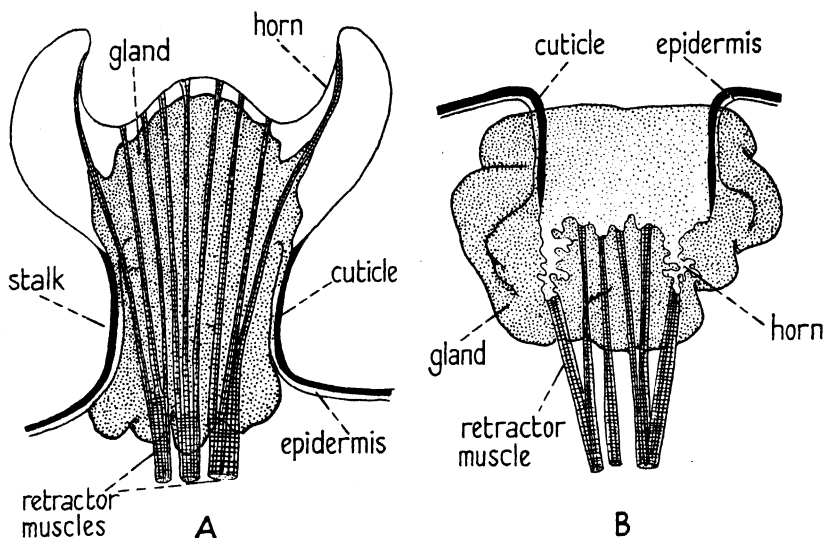


TEXT-FIG. 5. Géné's organ in *Ornithodorus moubata* (schematic). Optical sections of the organ in the everted (A) and retracted (B) condition.

between the gland and cuticle. The amount of secretion in the horns varies considerably: sometimes there is only a little at the tips and the gland then extends well into the base of each horn.

On retraction the entire organ is pulled through the narrow slit in the camerostome and turned inside out. The gland then comes to lie in a series of pleats inside the body-cavity, while the cuticle of the stalk and horns is contained in a highly folded state within the gland itself (Text-fig. 5B).

Géné's organ has a powerful system of retractor muscles which penetrate between the cells of the gland and run back through the stalk to insertions in the dorsal body-wall. Most of the distal attachments of these muscles are found on the cuticle between the horns, but each horn is provided also with a single retractor attached near the tip (Text-fig. 5B). These observations resolve the difficulty experienced by Robinson and Davidson (1914) in accounting for retraction. These authors, who studied only the invaginated organ in *Argas*

*persicus*, believed that the retractor muscles were attached only to the epidermis or gland ('hypodermal sac') and not to the cuticle ('chitinous sac').

The mechanics of the process of eversion have not been made out with certainty. Géné's organ in *Ornithodorus* is devoid of any intrinsic musculature capable of causing eversion. There is, therefore, a certain resemblance with the blow-fly ptilinum which also lacks intrinsic protractors (Laing, 1935). On the other hand, there are no externally visible signs such as pulsations of the cuticle which would suggest that Géné's organ, like the ptilinum, is inflated by locally increased blood-pressure. Robinson and Davidson held that eversion is brought about by the rapid secretion of fluid between the 'hypodermal' and 'chitinous' sacs. But this again is unlikely as the amount of secretion in the horns during normal oviposition appears to be no greater at the moment of eversion than at any other time between the successive delivery of eggs when the organ is retracted. It is more probable that a number of indirect muscles are concerned: the most important is certainly the depressor of the hypostome which is also inserted on the dorsal body-wall just posterior to the point of insertion of the gland retractor. This muscle appears to form a partial septum which, as it shortens and depresses the hypostome, impinges against the invaginated gland. It is noticeable that the organ can be everted with a much smaller application of pressure if the hypostome is held simultaneously in the depressed position.

Nuttall (1908) mentions that the inflated vesicle of Géné's organ in *Haemophysalis* is covered with minute punctuations which he took to be pits from which the secretion of the organ escaped. In *Ornithodorus* papilla-like processes are sometimes present near the base of the horns. Examination of serial sections showed, however, that these corresponded merely to the points of the muscle insertions. There is no specialized channel by which the secretion can escape through the cuticle of the organ. We consider this question in greater detail below.

### The Site of Wax Secretion on Géné's Organ

Although, under certain conditions, large external deposits of wax may be formed (p. 295), the points of secretion cannot easily be determined by inspection owing to the spreading propensities of the wax. The internal structure has, therefore, been examined in greater detail with the object of determining the site of transfer through the cuticle.

Sagittal and horizontal sections of whole organs fixed in the everted state show clearly that the gland is actually a specialized region of the epidermis which is continuous with the epidermis underlying the general body cuticle (Pl. I, fig. 2). There is no differentiation into 'hypodermal sac' and 'gland' such as Robinson and Davidson (1914) describe. At the base of the stalk the epidermal cells are relatively attenuated but at a point just proximal to the base of the horns the epidermis becomes greatly thickened and is folded inwards, so forming the pleats and convolutions of the gland.

The relations of the gland and epidermis to the cuticle are as follows. Over the basal region of the stalk the epidermis is closely applied to the cuticle, but near the point where the unspecialized epidermis passes into the thickened glandular tissue it becomes detached from the cuticle, thereby leaving a conspicuous lumen wherein the secretion of the gland accumulates (Text-fig. 5A; Pl. I, fig. 2). We have already stated that neither gland nor epidermis as a rule extends far into the horns. The walls of the horns were examined carefully in sections stained heavily with iron haematoxylin, but no trace of an inner cytoplasmic lining could be detected. There seems no doubt, therefore, that the secretion of the gland must be regarded as extracellular, accumulating between the cell wall and a part of the cuticle which is non-living.

The histology of the gland epidermis is shown in Pl. I, figs. 3 and 4. The component cells are columnar or wedge-shaped with well-defined cell boundaries. The nuclei lie near the margin of the cell abutting the haemocoele. In egg-laying females the gland cytoplasm is often distended with droplets, some of which appear to be on the point of discharge into the lumen (Pl. I, fig. 3).

The cuticle investing Géné's organ includes layers of smooth epicuticle and of endocuticle which pass without interruption into the cuticular layers of the general body-wall. The endocuticle is about 20 $\mu$ in thickness over the stalk region, but at those points where the secretion is stored it becomes very attenuated, only attaining a thickness of about 5 $\mu$ in the horns. Sections, cut at 6 $\mu$ and mounted in tap-water, were examined with an oil immersion objective for the presence of pore canals. Their approximate distribution is shown in Text-fig. 6B. Although there are numerous pore canals traversing the endocuticle of the stalk, they are more sparsely distributed near the point at the base of the horns where the epidermis becomes detached from the cuticle and pore canals are entirely absent from the endocuticle of the horns (Pl. I, fig. 7). In such regions, which are devoid of pore canals, it seems unlikely that the gland cells have any cytoplasmic connexions with the cuticle.

Previous work (Lees, 1947) has shown that in *Ornithodorus* the epicuticle of the general body cuticle is itself made up of successive thin layers of cuticulin, polyphenols, wax, and cement. Whether the pore canals penetrate the cuticulin layer, as in *Rhodnius* (Wigglesworth, 1947), is not known. If this is the case, their free ends are presumably covered over by the wax layer. The thin cement layer in turn forms an external protective covering over the wax. Now, once the cement has been laid down, no further deposits of wax can be secreted by the pore canals unless both cement and wax are removed (by abrasion, for example). Yet, as we have seen, the production of wax over Géné's organ takes place freely at intervals throughout the life of the egg-laying female. It is, therefore, important to know whether the cement layer extends on to the organ.

The presence of cement on the general body cuticle can be demonstrated by extracting the whole tick with cold chloroform for 30 minutes and staining

in 5 per cent. ammoniacal silver nitrate. The cement is insoluble in cold chloroform and protects the underlying wax from solution. Therefore the polyphenols beneath the wax layer cannot reduce the silver reagent where cement is present and these areas remain unstained. Since the epicuticle of Géné's organ also contains a polyphenol layer (covered in this case by the wax secreted by the organ itself) this method can also be used here for mapping the distribution of cement.

If a tick, with Géné's organ everted, is immersed in ammoniacal silver without previous extraction, there is no staining of the cuticle, showing that the wax layer is complete (Pl. I, fig. 5). On the other hand, if the tick is
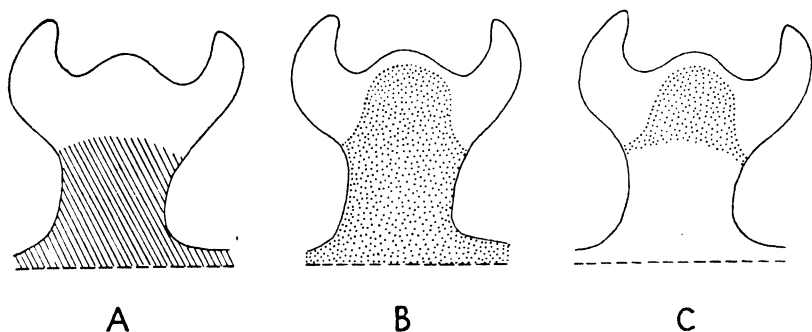


TEXT-FIG. 6. Géné's organ in *Ornithodorus moubata* showing the area covered by the cement layer (A), the distribution of the pore canals (B), and the probable site of wax secretion (C).

previously extracted for 30 minutes in cold chloroform, the cuticle round the base of the horns, and the horns themselves, stain intensely (Pl. I, fig. 6); nevertheless, like the general body cuticle, most of the stalk still fails to stain. This shows that the cement layer extends on to Géné's organ as far only as the base of the horns (Text-fig. 6A). The line of demarcation between the staining and non-staining areas is very sharp and corresponds with a line drawn round the circumference of the stalk which is well to the proximal side of a similar line marking the point of detachment of the epidermis from the cuticle. This result, which is of particular significance, was checked on sections of organs previously stained with silver (Pl. I, figs. 8, 9). There is a zone of varying width running round the circumference of the stalk between the bases of the horns where pore canals are present and cement absent (Text-fig. 6 c). In view of the marked chemical dissimilarity of the precursor from the horns and the wax from the surface of the cuticle (see p. 313), there seems no doubt that the former is first subjected to transformations by the living cells. If such is the case the wax must be secreted through those areas of the cuticle provided with pore canals (which contain, presumably, cytoplasmic processes extending from the living cells) and not through the non-living cuticle of the horns which is devoid of pore canals. According to this

view, the area shown diagrammatically in Text-fig. 6c also represents the site of the secretion of wax.

## PROPERTIES OF THE *ORNITHODORUS* WAXES

The waterproofing waxes examined by us have been derived from the following sources. The egg wax was obtained in its natural state from deposits occurring on the outside of Géné's organ; and small quantities of this wax were also extracted from vacated egg-shells. In addition, we have made some comparisons of the properties of the egg and cuticular waxes. The latter may be obtained in natural form from living ticks which have regenerated large quantities of this material after their cuticles have been thoroughly abraded with alumina dust (Lees, 1947), and the cuticular wax may also be extracted from cast nymphal skins.

### Properties of the Natural Waxes

The colourless transparent wax removed from Géné's organ has a somewhat syrupy consistency at 25° C. Small filaments of the wax, when warmed in a melting-point capillary, undergo rather indefinite optical changes between 36° and 46° C. and melt at 50–54° C. (Table 5). In view of the fact that the

TABLE 5. *Temperatures at which the natural waterproofing waxes of O. moubata, and the materials obtained from hot chloroform extracts, show optical changes*

| Material and source | Transitional changes ° C. | Melting-point, ° C. |
|---|---|---|
| Natural wax; Géné's organ . . . | 36, 40, 46 | 50–54 |
| White wax; egg-shell extract . . . | 56, 62, 72 | Above 100 |
| Yellow grease; egg-shell extract . . . | Indefinite | .. |
| Total egg-shell extract . . . . | 48 | 57 |
| Natural regenerated wax; adult cuticle . | 38, 53 | 65 |
| White wax; cuticle extract . . . | 75, 90 | 107 |
| Yellow grease; cuticle extract . . . | Indefinite | .. |
| Total cuticular extract . . . . | 70, 84 | 89 |

critical temperature of the egg is about 45° C. (p. 302), it is interesting to recall that Beament (1945) has found that increased evaporation from the cuticle of insects is associated more closely with changes in the optical properties of the wax, which occur below the melting-point, than with the melting-point itself.

The egg wax appears brilliantly luminous when viewed in polarized light and, as with other polycrystalline lipoids, exhibits no position of extinction between crossed nicols. It dissolves instantly in cold or hot chloroform or xylene but is insoluble in alcohol. Lumps of wax stain deeply with sudan black B but not with sudan III. The protein colour tests (xanthoproteic, Sakaguchi, ninhydrin, and Millon reactions) are all negative. The significance of this latter observation is discussed on p. 329.

The wax recovered from the surface of the cuticle after abrasion has entirely different physical properties. At 25° C. it is a hard white crystalline solid which shows no tendency to 'creep' along surfaces. As judged by the optical properties, transitional changes occur at 38° and 53° C. and the wax melts at 65° C. The critical temperature of the cuticle, as we have previously noted, is approximately 62° C. (Table 4).

### *Properties of the Extracted Lipoids*

The waterproofing substances from the cuticle were obtained by extracting the exuvia with chloroform. The cast skins were washed several times in cold water and dried in a desiccator. After repeated extraction in boiling chloroform under a reflux condenser, the solution was filtered through a hot-jacket funnel, evaporated to dryness at room temperature, and the total residue weighed.

The extracted material is not homogeneous, for two lipoids—a white waxy solid and a soft yellow grease—separate partially in the evaporating dish. Complete separation can be effected by treating the residue with acetone, in which only the grease is soluble. The white wax and the yellow grease are present in the total chloroform extract in the ratio of about 6:1 by weight.

The white crystalline wax has the following properties. Although the purified substance only melts to a clear liquid at 107° C., it undergoes optical changes, such as clearing, darkening, and loss of crystalline form, at lower temperatures (Table 5). These changes may be associated with crystalline transformations. The wax is soluble in boiling chloroform or benzene, and also in hot pyridine, but is sparingly soluble in the cold. It is insoluble in water, hot or cold alcohol, and hot or cold acetone. There are some indications that the material is capable of forming an oriented layer. Thus, if a crystal of the wax is dropped on to the surface of boiling water it forms a fine film which may be obtained by dipping a glass-slide through the surface; and this film has similar properties to the crystalline material save that it is now wetted by 50 per cent. alcohol. The white wax is not stained by either of the sudan stains, or by Ciba's B.Z.L., nor is it darkened by osmium tetroxide.

The second component, the yellow grease, is readily soluble in cold lipophilic liquids, including acetone and absolute alcohol; it stains with sudan III and with sudan black B and darkens with osmium tetroxide. The optical changes with temperature are indefinite.

Hot chloroform extracts of egg-shells (separated first from the larval exuvia) also yielded two fractions comprising white wax and yellow grease in approximately the same proportions as in the cuticle extracts. Their properties also appear to be very similar (for temperature changes see Table 5).

It would appear that the white wax may be a mixture of very long chain paraffins, acids and esters, while the grease may be composed of much shorter molecules, with unsaturated bonds along the chain.[1] In the natural waxes—the regenerated material from the cuticle or the wax from Géné's organ—the

[1] Private communication from Prof. A. C. Chibnall, F.R.S.

two lipoids seem to be present in some stable form of association, although this may not necessarily be of a strong chemical nature. This state does not appear to be automatically reconstituted when the two substances have been extracted with lipoid solvents. Nevertheless it is noteworthy that when the two components derived from the cuticular extracts are intimately mixed, the resulting melting-point of the mixture is considerably lower than that of the purified white wax and approaches the melting-point of the natural regenerated cuticular wax (Table 5).

### The Spreading Properties of the Egg Wax

*Spreading on the Egg.* Since a comparatively small part of the egg-shell appears to come in contact with Géné's organ during the normal oviposition movements (see p. 295), the newly secreted wax must be capable of spreading over the surface of the shell. The spreading properties of the wax were therefore tested in *O. moubata*. Unwaterproofed eggs were obtained as usual by allowing ticks with Géné's organ covered to deposit egg-clusters in damp air; and the wax was obtained from ticks kept continuously at 15° C., organs bearing massive deposits of wax being selected for use. Each egg was removed individually from the egg-mass with forceps, brought into contact with the wax on the everted organ, and manipulated so that the wax was thoroughly spread over the required surface. Sometimes the eggs of a batch were completely smeared with wax; sometimes, in order to test the spreading powers, approximately half the surface was smeared. After treatment the eggs were placed on damp filter-paper for 2 hours and were then exposed in a watch-glass to dry air at the same temperature. The control eggs, which remained unwaxed, were always drawn from the same egg-mass.

TABLE 6. *The effect of smearing unwaterproofed eggs of* O. moubata *with wax taken from Géné's organ of the same species*

The rate of shrivelling was observed in dry air

| Female no. | Treatment | Temperature, ° C. | No. of eggs | No. of eggs shrivelled after | | |
|---|---|---|---|---|---|---|
| | | | | 30 min. | 4 hrs. | 24 hrs. |
| 1 | None | 20 | 20 | 20 | .. | .. |
| | Wholly smeared | | 20 | 6 | 11 | 16 |
| 2 | None | 25 | 20 | 18 | 20 | .. |
| | Half-smeared | | 20 | 0 | 0 | 3 |
| 3 | None | 30 | 20 | 13 | 20 | .. |
| | Half-smeared | | 20 | 0 | 0 | 0 |
| 4 | None | 19 | 20 | 19 | 20 | .. |
| | Half-smeared | | 20 | 20 | .. | .. |
| 5 | None | 19. | 20 | 2 | 4 | 10 |
| | Half-smeared | | 20 | 2 | 2 | 4 |

The results, which are given in Table 6, show that the rate of shrivelling is considerably reduced when the eggs are completely smeared with wax. Nevertheless, in spite of the fact that the amount of wax transferred to each

egg is probably greatly in excess of the normal quantity, and that a greater surface area of the shell is brought into contact with the wax than during the natural manipulation by Géné's organ, the final degree of impermeability is always inferior to that of the egg when waterproofed naturally.

Results with half-smeared eggs proved to be very variable. In some egg batches (e.g. females 2 and 3) the permeability was greatly reduced, indicating that considerable spreading of the wax had taken place. In others (e.g. female 4) the eggs shrivelled as rapidly as the controls, thus providing no evidence of effective spread. No certain correlation between temperature and the rate or efficiency of waterproofing could be established. But the waterproofing effect is usually more pronounced if eggs are selected whose shells, even before the application of wax, are relatively impermeable (e.g. female 5, Table 6).

No evidence of spread was obtained with smaller areas of contact. A number of eggs, each with a large lump of wax adhering at one point, shrivelled as rapidly as the controls at an incubation temperature of 30° C. The limited spreading powers of the wax are also shown by its inability to spread effectively from egg to egg. A number of unwaterproofed eggs were, for example, placed in contact with normal waterproofed eggs or were thrust into the centre of a normal egg-mass. Yet these always shrivelled on subsequent exposure to dry air. If a female tick is interrupted in the process of oviposition, Géné's organ never bears any large wax deposits although there is always a film of wax bounding the epicuticle (as can be shown by the failure of the epicuticle to stain with silver from the outside). This film of wax is also incapable of limiting evaporation from unwaterproofed eggs if these are brought into intimate contact with the organ. It would appear, therefore, that the production of wax by Géné's organ may, to some extent, be co-ordinated with the delivery of the egg by the vaginal 'ovipositor'.

An additional factor influencing the spread of wax is probably the smoothness of the substratum. It was noted that waterproofing was invariably poor if any fungal hyphae were growing on the surface of the shell.

It is possible also to reduce the permeability of *Ixodes* eggs by applying the egg wax of *Ornithodorus*. Eggs laid without Géné's organ (and therefore already partially covered with *Ixodes* wax) were completely smeared with wax on an everted organ of *Ornithodorus*. Water loss was then found to be slightly less than from normal *Ixodes* eggs. The following are examples of the results obtained with batches of 20 eggs laid by the same tick. In a normal batch, laid with Géné's organ, 4 eggs had shrivelled after 4 hours, and 20 after 24 hours, in dry air at 30° C. In a second batch, laid with Géné's organ covered, every egg was completely dried out within 4 hours. In a third, laid without Géné's organ, but wholly smeared with *Ornithodorus* wax, no eggs had shrivelled after 4 hours and only 15 after 24 hours. The effectiveness of the waterproofing is, however, hardly comparable with that exhibited by the normal *Ornithodorus* egg.

*Spreading on Membranes.* Beament (1945) has shown that extracted insect cuticular waxes may be deposited on prepared membranes from chloroform

solutions. Provided that the surface film is continuous the membrane then reproduces many of the physical properties (e.g. critical temperatures) of the normal insect cuticle from which the wax was obtained. Experiments were carried out to determine whether natural or extracted waxes of *Ornithodorus* could also be used in a similar manner. The membranes consisted of lipoid-free butterfly wings, chloroform-extracted wings of *Rhodnius prolixus*, and large wax-free nymphal skins of *O. moubata*. These were mounted in holders of the type described by Beament (loc. cit.); the surface area exposed was adjusted so as to range from 3 to 50 mm.². The sources of the waxes and the methods of application were as follows: (i) Natural wax from Géné's organ was smeared in the centre of the membrane. (ii) Wax from Géné's organ was deposited on the membrane from cold chloroform. (iii) The total chloroform extract from egg-shells, the acetone-soluble fraction (yellow grease), and the chloroform-soluble fraction (white wax) were deposited separately from cold and hot chloroform. (iv) The same procedures were also carried out with regenerated cuticular wax and with the cuticle extract.

Because of several initial failures, each waxed membrane was subsequently heated in an oven at different constant temperatures which varied from 30° to 70° C. It was hoped that heat would assist spreading in an uneven deposit of wax and promote an alinement of the wax molecules more consistent with impermeability. The steady water loss from each membrane was recorded at room temperature before and after heat treatment.

In none of the membranes could any reduction in permeability be detected, with the exception of the following. A lipoid-free *Ornithodorus* nymphal skin, 3 mm. in diameter (and therefore about three times the surface area of an *Ornithodorus* egg) had an initial permeability at 20° C. of 20 mg./sq.cm./hr. After smearing it with the natural wax from Géné's organ and heating to 50° C. the rate of transpiration at 20° C. fell to 5 mg./sq.cm./hr. Nevertheless, this membrane was still too permeable for testing the critical temperature. It seems clear from these results that the natural egg wax must spread relatively slowly, even at high temperatures. And chloroform appears to have the effect of separating the wax system into two components neither of which can be redeposited on a membrane in the form of a waterproofing layer.

*Spreading on Water.* The spreading pressures of substances on water may be compared in the apparatus described by Adam (1945). Water is introduced into a large funnel immersed in a water bath and is flushed through until the surface is clean. This is then covered with a fine film of lycopodium powder which will indicate changes in the surface when any foreign material is added.

If Géné's organ is everted and applied to the centre of the prepared water surface, the powder is displaced slowly and a film of wax spreads from the organ over a small area. We have noted that the wax is not very mobile at room temperatures; the secretion spreads more rapidly if the temperature of the water surface is raised, but it appears to cover only the same small area.

If the eggs of *O. moubata* are dropped on to the prepared water surface, slow spreading over a limited area takes place at all temperatures between

20° and 60° C. This phenomenon was obtained with newly laid eggs and with those about to hatch. It is obvious that the waterproofing lipoid is not completely bound or otherwise immobilized on the shell surface.

Material spreads much more rapidly from the surface of eggs of *I. ricinus* at room temperature and extremely rapidly at higher temperatures, but the waxy film again fails to cover a very large area. By comparison with the spreading powers of the waterproofing grease from the cockroach cuticle (Beament, 1945), the surface activity of the tick waxes is slight. Such spreading powers as the egg waxes possess seem to be associated with the presence of the acetone-soluble material (the yellow grease) which spreads actively. The white wax is devoid of spreading properties.

### The Amount of Wax present on the Egg

The average weight of waterproofing material on the egg can be derived from a knowledge of the weight of extracted material and the total number of egg-shells extracted. Assuming the relative density to be 0·96 g./c.c. (Lewkowitsch and Warburton, 1921), the average volume of wax covering these eggs can be found. The approximate surface area of the egg may be arrived at from camera lucida drawings and geometric considerations. The thickness of the extracted materials, if spread evenly over the surface of the egg, can then be calculated.

The thickness of the wax layer in the *Ornithodorus* egg was found to be approximately 0·47 $\mu$ and in the *Ixodes* egg 1·76 $\mu$. On the other hand, the thickness of the cuticular wax layer in the nymph of *Ornithodorus* is no more than 0·29 $\mu$.
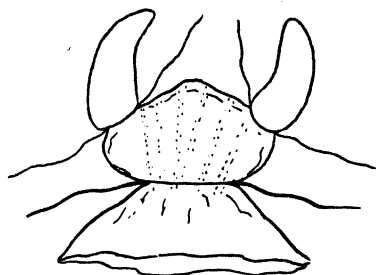
It will be noted that in both species of ticks the waterproof covering of the egg is considerably thicker than the wax layer encountered on the inside of the *Rhodnius* egg-shell (Beament, 1946b); and it is also of greater thickness than the waterproofing layers on the cuticles of several insects (Beament, 1945), and of the nymphal tick itself. The calculation made above assumes an even thickness of wax on the egg. In fact, visual inspection shows that this is by no means always true, for eggs may often be seen in the egg-masses of *Ornithodorus* which have definite mounds of wax adhering at one or more points. Nevertheless, the figures may be taken to indicate that each egg receives a slight excess of wax.

### The Nature of the Contents of Géné's Organ

The nature of the glandular secretion of Géné's organ is of considerable interest as it is evident that the secretion is associated in some way with the production of wax on the outside of the organ and may in fact be the wax precursor.

The contents of the horns, a clear refractile liquid, can be examined *in situ* with the 1/12th oil immersion objective. Apart from pieces of tissue (derived from fragmentation of the gland), which are abundant in some organs but completely absent in others, no particles or droplets are visible within the limits of resolution.

It is possible to collect small quantities of the liquid by applying a fine ligature of silk thread at the base of each horn and another stouter ligature round the base of Géné's organ, then cutting it free and pricking each horn separately on to a glass slide. The method is illustrated in Text-fig. 7. By careful selection of the ligatured organs it is possible to obtain the secretion virtually free of organic debris from the gland.



TEXT-FIG. 7. The method of ligaturing the horns of Géné's organ in *Ornithodorus moubata*.

The liquid from the horns can be shown to contain water by pricking the ligatured horn on to a small crystal of anhydrous cobalt chloride in a dry atmosphere. The crystal instantly turns red. If the water is allowed to evaporate after pricking on to a glass slide, a speck of solid material is left. This is perfectly transparent and is sometimes slightly brittle, but has a waxy feel when scratched with a needle. On adding droplets of warm or cold water the material redissolves instantly. Xylene and chloroform, which are excellent solvents for the wax deposits on the outer surface of the horns (p. 308), fail even to wet it.

Protein or protein derivatives are present in the contents of the horns. The following tests, which are summarized in Table 7, were performed on specks

TABLE 7. *Some properties of the material isolated from the gland of Géné's organ in* O. moubata

| Test | Contents | Observation |
|---|---|---|
| Water   .         .        .        . | Solid | Dissolves instantly |
| Arginine reaction  .        .        . | Aqueous solution | Strongly positive |
| Ninhydrin   .        .        .        . | ,,          ,, | Positive |
| Xanthoproteic reaction   .        . | ,,          ,, | Negative |
| Aldehyde reaction  .        .        . | ,,          ,, | ,, |
| Millon's reaction  .        .        . | ,,          ,, | ,, |
| Ethyl alcohol   .        .        . | ,,          ,, | White flocculant precipitate |
| Phosphotungstic acid   .        . | ,,          ,, | ,,          ,,          ,, |
| Phosphomolybdic acid   .        . | ,,          ,, | ,,          ,,          ,, |
| Picric acid   .        .        .        . | ,,          ,, | Yellow precipitate |
| Aluminium chloride   .        . | ,,          ,, | Solution remains clear |
| Sodium citrate   .        .        . | ,,          ,, | ,,          ,,          ,, |
| Adjust to pH 8 or to pH 3   . | ,,          ,, | ,,          ,,          ,, |
| Sudan III and sudan black B . | Solid | No staining |
| Heat   .        .        .        .        . | ,, | Does not melt at 100° C. |
| Crossed nicols   .        .        . | ,, | Faintly luminous |

of the material redissolved in distilled water. The ninhydrin reaction is positive indicating the presence of amino groups. The Sakaguchi reaction for arginine, which was performed by adding successive droplets of 5 per cent.

NaOH, 1 per cent. alcoholic α-naphthol, and 10 per cent. sodium hypochlorite, is invariably strongly positive. Other colour reactions, including the sulphur reaction for cystine, the aldehyde reaction for tryptophane, the Millon and xanthoproteic reactions, are negative. The material must, therefore, be poor in tyrosine and tryptophane.

The protein in the horn contents is rapidly coagulated by the addition of ethyl alcohol forming a flocculent white precipitate. It is not precipitated by concentrated nitric acid in which the solid dissolves to yield a clear solution; neither is it readily heat-coagulable. If the solid is warmed on a slide at 100° C. for 1 hour, it immediately redissolves on the addition of water and this also occurs after warming in the presence of a droplet of 1 per cent. acetic acid. With stronger heating over a flame the solid material is gradually coagulated and fails to redissolve completely in water. It does not melt.

The protein is readily precipitated in aqueous solution by alkaloidal reagents such as picric, phosphotungstic, phosphomolybdic, and trichloracetic acids and fails to redissolve in water.

We seem to be dealing here with a protein rich in di-amino-acids. The high arginine content, solubility in water, and the resistance to coagulation by heat suggest some affinities with the protamines or histones. The reactions detailed above could not all be ascribed to the presence of free amino-acids, peptones, or proteoses (which might conceivably be present as emulsifying agents).

With the minute quantities of material available for tests, it has not been possible to isolate from the contents of the horn any material whose identity with the wax could be proved beyond doubt. Two observations do, nevertheless, lend some support to the view that the horns contain the wax precursor. First, a speck of the solid material treated with 5 per cent. trichloracetic acid becomes difficult to wet with water, the surface developing at the same time a glistening waxy appearance. However, although the coagulum is now readily wetted by chloroform little appreciable solution takes place. Secondly, the appearance in polarized light also affords some slight evidence of the presence of long chain lipoids. A small patch of material from the horns is isotropic when viewed normal to the surface but particles scraped up on a needle are decidedly birefringent in all quadrants. The material is therefore polycrystalline, as the wax itself is (p. 308).

Certain facts suggest that the wax is not present in the horns as a simple emulsion. For example, the horn contents are not readily precipitated by changes in the reaction of the medium. If the solution is adjusted to pH 8 or pH 3 by adding, respectively, droplets of borate or phthalate buffer, no trace of milkiness develops. Further, the solution remains perfectly translucent in the presence of excess polyvalent ions (e.g. calcium, aluminium, citrate).

The horn contents have no powers of emulsifying waxes in bulk. The absence of any effective agent can be shown by pricking a ligatured horn on to a small lump of wax removed from the outer surface of Géné's organ. No visible emulsification takes place and the watery contents of the horn fail even to wet and mix with the wax.

## The Morphology of the Female Genital System in Ticks

Several detailed accounts of the morphology and histology of the genital system are available. Among Argasidae we have descriptions for *Ornithodorus savignyi* (Christophers, 1906) and *Argas persicus* (Robinson and Davidson, 1914); and among Ixodidae for *Ixodes ricinus* (Samson, 1909) and *Dermacentor andersoni* (Douglas, 1943). Some of the salient features will be recapitulated
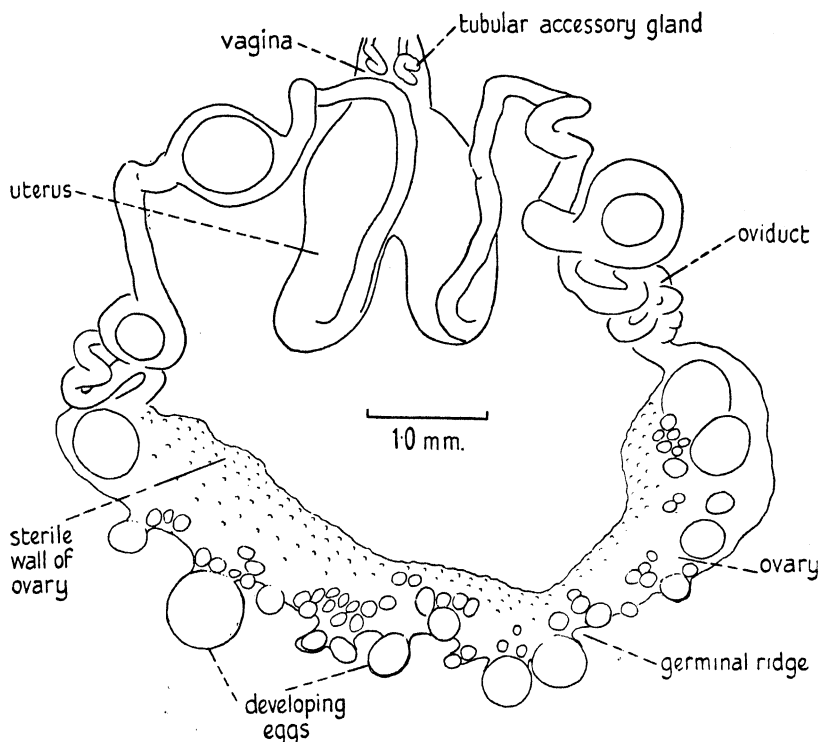


Text-fig. 8. Female genital system in *Ornithodorus moubata*.

below. Our inquiry has, however, been directed principally towards two specific questions. In the first place, a rather more detailed treatment of the development of the ova in *O. moubata* has been a necessary preliminary for an extended examination of the structure and properties of the egg-shell. And secondly, the genital tract in *I. ricinus* has been re-examined in an attempt to discover the site of application of wax to the egg, for, as we have shown, a waxy covering is already present on the egg before it is received by Géné's organ.

### The Genital Tract and Ovary in O. moubata

The genital system of an egg-laying female is shown in Text-fig. 8. There is, as in all ticks, a single garland-like ovary which passes at each end into the

long coiled oviducts. The posterior wall of the ovary is studded with developing ova of all sizes, but the anterior wall is sterile and consists of undifferentiated cells only. This type of tubular ovary with a parietal germarium occurs throughout the Arachnida and may represent an archaic feature of their organization. The tick ovary, indeed, closely resembles that of the Onycophora (Manton, 1938).

The oviducts pass into the large sac-like uterus from which arises the short vagina leading to the genital aperture. Robinson and Davidson (1914) distinguish two sections in the vagina, a posterior or cervical region, provided with a thick investment of muscles, and a short vestibular region without circular muscles. Two small tubular accessory glands enter the vagina near their junction.

The general course of development of the eggs appears to be as follows. The ova arise from small undifferentiated cells lying in the germinal ridge. This part of the ovary is itself invested externally by a fine hyaline non-chitinous membrane, the tunica propria. As the egg grows in size it begins to stretch this membrane and projects outwards from the surface of the ovary (Text-fig. 9A). By the time a diameter of 100 $\mu$ has been attained the egg has acquired a stalk or funicle whose walls are made up of a single layer of undifferentiated epithelial cells (Text-fig. 9B). These cells stain blue in Mallory, whereas the cytoplasm of the immature egg acquires a strong affinity for acid fuchsin. Having attained a diameter of about 500 $\mu$ the egg is finally ovulated into the lumen of the ovary. This process appears never to have been observed, but there is little doubt that, as Christophers (1906) implies, the continuous growth of the egg must impose an increasing strain on the elastic tunica propria so that eventually pressure forces apart the cells of the funicle and the egg passes through.

The appearance of the shell can first be detected when the egg is 25 $\mu$ or less in diameter. According to Nordenskiöld (1909) the shell in *I. ricinus* is first laid down in the form of adjacent granules which coalesce to form a continuous membrane, the last region of fusion being in the region of the funicle. This author held therefore that the funicle played some part in secreting the shell; and also that nutritive material for the developing egg is drawn from the lumen of the ovary via the funicle, as well as from the haemolymph through the tunica propria. In our sections of the ovary in *Ornithodorus* the shell first appears as an exceedingly tenuous pellicle which is continuous over the funicular region, as elsewhere. It is to be doubted whether the funicle plays any part in secreting the shell for the two are not firmly attached to one another. This can be shown if the ovary is dissected in saline and the tunica propria is slightly torn with a needle. Developing ova of all sizes then roll out freely into the dissecting-dish. Yet the shell clearly increases both in surface area and in thickness as the ovum grows in size within the tunica propria. It is apparent from this that the shell must be secreted by the oocyte itself. Among the insects the term 'chorion' is best reserved for those parts of the shell secreted by the follicle cells (Beament, 1946a). We shall not therefore apply this term to the tick egg-shell.

As the egg passes down the ovary into the oviduct it absorbs water, finally reaching a diameter of about 800 $\mu$. The eggs are stored for a short while in the uterus before being laid.

The outer surface of the tick egg is completely smooth and featureless; there is no micropile. Robinson and Davidson (1914), finding sperms only as
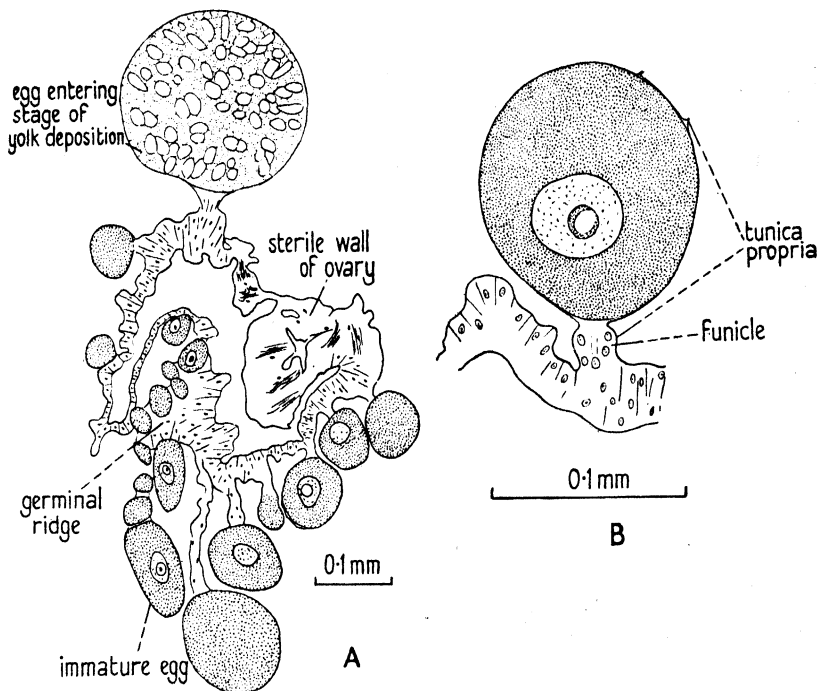


Text-fig. 9. The structure of the ovary in *Ornithodorus moubata*: A, transverse section through a mature ovary; B, section through an immature egg developing in the germinal ridge.

high as the upper oviduct in *A. persicus*, suggested incorrectly that the shell was laid down by the oviduct shortly after fertilization. But we have seen that in *O. moubata* the shell rudiment is acquired very early in development. How then is the egg fertilized?

In this species, as in other Argasidae, the uterus also serves as a receptaculum seminis, often containing as many as a dozen spermatophores. As these are ruptured, the sperms, which are relatively gigantic objects some 400 $\mu$ in length, make their way up the oviducts, presumably by means of the slow gliding movements of which they are capable. The genital tracts of fertilized ticks, which were dissected in Ringer and examined with a 1/6th objective, often contained isolated sperms in the lumen of the ovary as well as large clusters of sperms in the upper oviducts. There would appear to be no

escape from the conclusion that the oocyte is fertilized *in situ* within the germinal ridge before the rudiments of the shell are laid down at the beginning of the growth phase.[1] It is of considerable interest in this connexion that Christophers (1906) has figured a developing egg in *O. savignyi* only about one-quarter grown and yet containing the clearly recognizable remains of a spermatozoon. In spite of careful search, however, we have found no similar traces within the eggs of *O. moubata* at any developmental stage. Nevertheless, in view of the resistant nature of the egg-shell (p. 322), it is highly improbable that sperms could enter the egg after the deposition of the shell has begun. Uterine eggs are usually surrounded by spermatozoa but they never penetrate the shell.

### The Genital Tract in Ixodes ricinus

The ovary in this Ixodid is much longer than in *O. moubata* and contains greater numbers of developing eggs. On the other hand, the general course of ovulation and of egg development is probably very similar and need not be described here.

There are, however, important morphological differences in the distal regions of the genital tract. There is no uterus in *I. ricinus* and the two oviducts join to form a short common oviduct leading directly to the vagina (Text-fig. 10). Further, there are two types of accessory glands: short, club-shaped or tubular glands, probably homologous with the tubular glands in *Ornithodorus*, and a larger gland whose numerous irregular lobes are disposed on the dorsal and lateral sides of the vestibular portion of the vagina. The latter is without a homologue in *Ornithodorus*. Douglas (1943) refers to this lobed accessory gland in *Dermacentor andersoni* as a receptaculum seminis and regards it as comparable with the uterus in Argasidae—a term which, in his opinion, is a misnomer. In *Ixodes*, however, the lobed accessory glands never contain spermatophores; on the contrary, these seem to be passed directly into the common oviduct.

The general arrangement of these organs is shown in the sagittal section (Text-fig. 11). The cervical and vestibular regions of the vagina, with the tubular accessory glands opening near their point of junction, can again be distinguished. The entire vagina is lined by thin cuticle which is continuous with the general body integument. Over the vestibular region, epicuticle and a thin layer of endocuticle is present; it is highly folded and in places bears minute recurved spines. In the cervix the intima is reduced to a tenuous layer of epicuticle which closely follows the contours of the vaginal cells. The latter are closely invested with a deep layer of circular muscle-fibres which, by their contraction, are probably responsible for the prolapse of the vagina during oviposition (Pl. I, fig. 12).

We have shown that the eggs dissected from the oviducts are completely unwaterproofed but that they acquire an incomplete layer of wax on the

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[1] The precocious uptake of sperms by the young oocyte is described in *Peripatopsis* by Manton (1938), but here the function is stated to be that of providing nutriment for the growth of the ovum.

surface of the shell during their passage down the vagina. The source of this wax may therefore be (i) the cells lining the cervical region of the vagina, (ii) the tubular accessory glands, or (iii) the lobed accessory glands. Their histology affords convincing evidence of the close affinity of one of the organs —the lobed accessory glands—with Géné's organ.
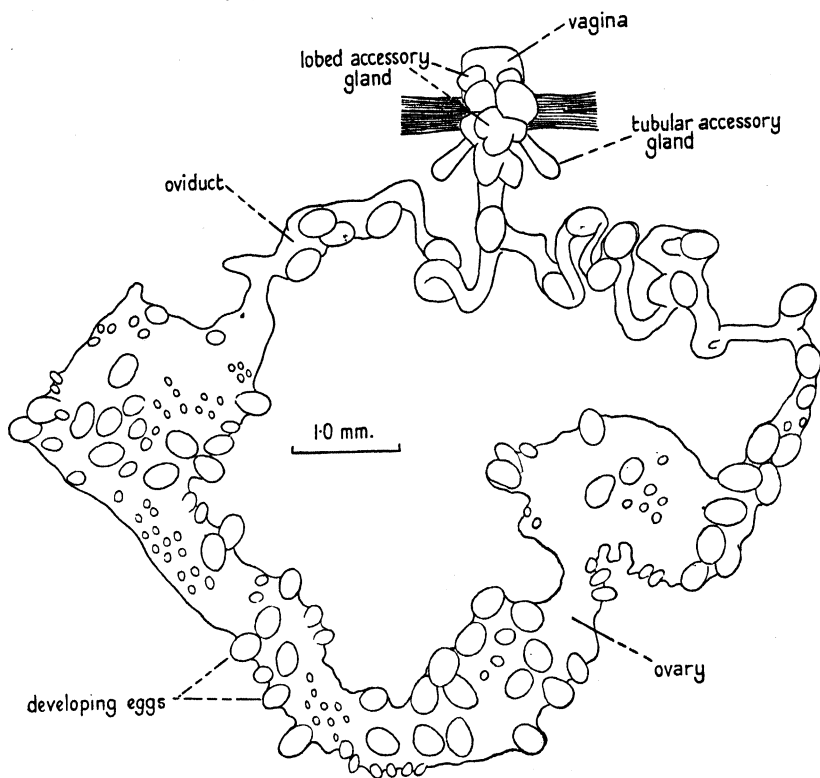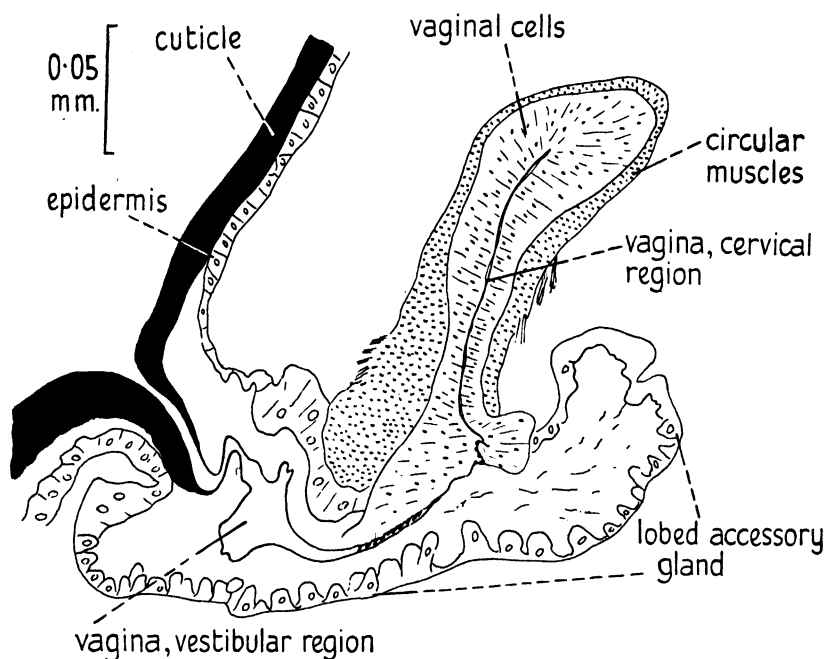


TEXT-FIG. 10. Female genital system in *Ixodes ricinus* towards the close of oviposition.

A section through the wall of one lobe of Géné's organ is shown in Pl. I, fig. 10. The histological appearance is completely different from that of the corresponding organ in *O. moubata*. The gland-cells are very large by comparison, are often widely spaced from each other, and are irregular or polygonal in shape. The cytoplasm is dense and deeply staining, the nuclei are large, and there is a well-developed 'honeycomb' border. Not infrequently an irregular meshwork of fibres extends from the cell margin into the gland lumen.

The tightly packed columnar epithelial cells lining the cervix have poorly defined cell boundaries and small nuclei disposed at different levels in the cell (Pl. I, fig. 12). As already mentioned, the cuticular intima maintains close contact with the free borders of the cells.

The tubular accessory glands are built up of wedge-shaped cells bordering a narrow central lumen (Pl. I, fig. 13). Their cytoplasm usually contains large droplets of colloid which stain a pale grey with iron haematoxylin. The lumen appears to open freely into the genital tract.

On the other hand, the histological appearance of the lobed accessory gland (Pl. I, fig. 11) and the gland of Géné's organ is so similar that the two cannot



Text-fig. 11. Median sagittal section through the female genital tract in *Ixodes ricinus*.

easily be distinguished unless the arrangement of the lobes is followed carefully in serial sections. Probably, therefore, the former is responsible for secreting the wax. It may be noted that this gland bears the same relation to the vaginal cuticle as does the gland of Géné's organ to the cuticle of the horns, for the lumen of the accessory gland is partitioned off from the genital tract by the cuticular lining of the vestibule, which is in contact with the cells only near the margins of the gland. The accessory gland—also like Géné's organ—represents an intucked region of the epidermis which has become specialized for a particular glandular function.

An additional point of interest is the absence of a homologous organ in *O. moubata*. In this species, as we have seen, there is no evidence that waxes are secreted on to the egg during its passage down the vagina.

## THE STRUCTURE AND CHEMISTRY OF THE EGG-SHELL IN *O. MOUBATA*

Three distinct layers can be distinguished in the shell of the newly laid egg: (i) an inner 'shell layer'; (ii) an incomplete layer of granules with reducing properties; and (iii) the outer covering of wax which is applied by Géné's organ (Text-fig. 12A). A fourth layer, the 'inner membrane' (iv), is secreted after the egg has been incubated for 2–3 days (Text-fig. 12A, B). The properties of these layers may now be outlined; those of the wax have already been described.

*The Shell Layer.* One-day-old eggs were punctured, freed from yolk in distilled water, dried, and extracted in boiling chloroform to remove soluble
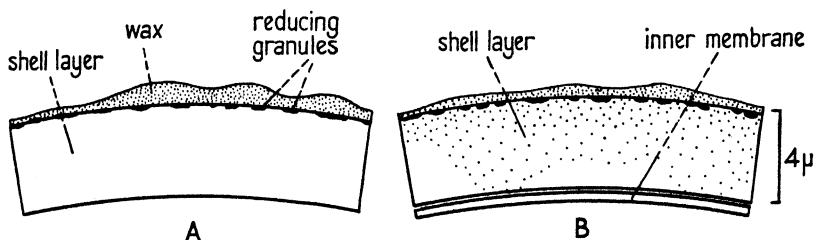


TEXT-FIG. 12. Diagram illustrating the structure of the egg-shell in *Ornithodorus moubata*. A, 0–1 days after laying. B, After incubation for 6 days at 25° C.

lipoids. The observations on this material were repeated on shells of eggs dissected from the ovaries at various stages of growth and on eggs which had been laid without establishing contact with Géné's organ. The latter are, of course, free from wax and need not be extracted first in chloroform.

The lipoid-free shell layer is a uniform membrane with striking elastic properties, smooth on its outer and inner surfaces and 3–4 $\mu$ in thickness. It is colourless, the tint of the egg depending entirely on the pigment in the yolk. There are no pore canals.

The shell layer contains no chitin but it gives a strong xanthoproteic reaction and turns pink when heated with Millon's reagent. The ninhydrin reaction is negative and it fails to stain with cold and hot *p*-benzoquinone. The shell material stains deeply and rapidly with basic and acid fuchsin, borax carmine, haematoxylin, and picric acid. It fails to take up colour from alcoholic solutions of sudan III or sudan black B, nor does it darken with osmium tetroxide.

The shell membrane is insoluble in cold concentrated nitric acid, but dissolves on heating, leaving a granular residue; no oil is released. It is partially soluble in cold concentrated nitric acid saturated with potassium chlorate and on warming dissolves completely with the evolution of gas. The shell material dissolves readily in hot potash and is also rapidly broken down by 14 per cent. sodium hypochlorite.

This evidence shows that the shell layer is composed of a protein which, unlike the 'cuticulin' of *Rhodnius* epicuticle (Wigglesworth, 1947), does not incorporate a lipoid.

*The Granular Layer.* If whole eggs, laid without Géné's organ, are immersed for 15 minutes in 5 per cent. ammoniacal silver nitrate, the outer surface of the shell layer, after mounting, is seen to be covered with scattered granules and granule aggregates which have stained a deep reddish-brown (Pl. I, figs. 14, 15). The nature of these granules is uncertain for they are also stained by 1 per cent. silver nitrate (but not by silver nitrate in the presence of nitric acid). They appear on the shell layer shortly before the ovulation of the egg. Two further points are worthy of note. First, the shell layer has the appearance of a material which is partially tanned. Thus the shells of eggs newly ovulated into the lumen of the ovary are decidedly flabby and elastic, but they lose much of this elasticity and become much more rigid as they pass down the genital track into the uterus. Secondly, the reducing granules of the tick egg, although not identical with the polyphenol granules which constitute the substrate for the waterproofing layer in the *Rhodnius* egg-shell (Beament, 1946a), occupy the same functional position with respect to the wax layer.

*The Inner Membrane.* After incubation for 2–3 days at 25° C. a further layer, also secreted by the embryo, is added to the inner surface of the shell. It is easily demonstrated if empty egg-shells are treated with sodium hypochlorite solution: this dissolves the shell layer, leaving a tenuous transparent membrane which retains the same outline. The inner membrane is absent in the one-day egg.

The chemical properties of the inner membrane are mainly negative. It gives no definite colour with Millon's reagent and the xanthoproteic and ninhydrin reactions are negative. It does not contain chitin. The material is insoluble in cold and hot nitric acid but eventually soluble in hot nitric acid saturated with potassium chlorate. No oil is liberated when solution takes place. It is insoluble also in sodium hypochlorite and aqueous potash, but dissolves in fused potash. Water-soluble dyes such as borax carmine, basic or acid fuchsin fail to stain the membrane and it remains uncoloured after treatment with *p*-benzoquinone and ammoniacal silver nitrate.

The resistance of this material to attack by chemical agents therefore recalls the properties of the fertilization membrane of the *Rhodnius* egg (Beament, 1948a).

## PERMEABILITY OF THE EGG-SHELL

Eggs which have received no wax from Géné's organ are highly permeable to water and shrink rapidly in dry air (p. 296). As might be expected, unwaterproofed eggs of *O. moubata* also show notable swelling properties when immersed in distilled water. Developing ova taken from the germinal ridge of the ovary swell to nearly three times their normal diameter in 5 minutes before bursting (Text-fig. 13D, E); those from the uterus, because of their more rigid shells, swell more slowly, a hyaline zone appearing between

the yolk and the shell (Text-fig. 13A, C). Swelling may finally cease altogether.

We have already noted that water loss from the one-day egg, waterproofed in the normal manner by Géné's organ, is enormously hastened by extraction in cold chloroform. This statement, however, does not necessarily hold good for eggs in a later stage of incubation. It has been found indeed that the effect on transpiration gradually diminishes as the period of incubation lengthens and that the eggs also develop increasing resistance to treatment with abrasive dusts. Some of the results obtained with eggs of known age are set out in Table 8.

After incubation for 3 or 4 days at 25° C., short extractions (1 min.) in cold chloroform, which undoubtedly dissolve all the wax from the surface of the
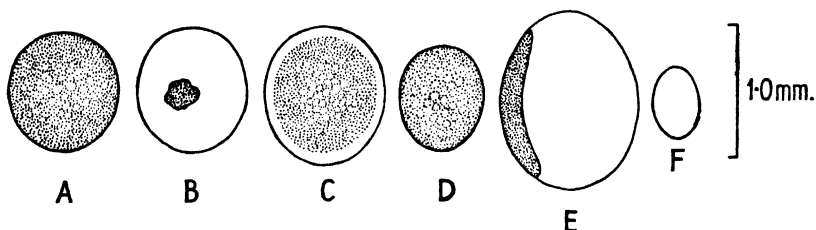


TEXT-FIG. 13 A–E. Eggs of *Ornithodorus moubata* after various treatments: A, normal egg; B, waterproofed egg extracted for 4 hrs. in cold chloroform; C, unwaterproofed egg from uterus after 30 min. in distilled water; D, egg dissected from germinal ridge; E, a similar egg after 5 min. in distilled water. F, egg of *Ixodes ricinus* in outline.

shell, leave the egg almost as resistant to desiccation as previously. Longer periods of extraction have a noticeable effect on older eggs, while the longest extraction (4 hrs.) completely destroys the waterproof properties, even in those eggs which are on the point of hatching. Penetration is particularly noticeable after 4 hours for chloroform gradually displaces the water in the egg and, without any accompanying change in the egg volume, forms a refractile zone round the shrivelled yolk (Text-fig. 13B). In a comparable manner, the one-day egg is highly sensitive to abrasion and shrivels immediately in dry air after it is rubbed with alumina dust or even if it is merely sprinkled with dust: whereas the older eggs again develop considerable resistance to this treatment, sometimes being seemingly unaffected by the most thorough abrasion (Table 8).

Two explanations of this phenomenon seem possible. On the one hand, the mobile wax applied by Géné's organ to the outside of the shell may gradually sink in and impregnate the shell layer; it would then still be liable to extraction by prolonged treatments with chloroform but would be inaccessible to the influence of abrasive dusts. Or, alternatively, one could postulate the deposition of a secondary waterproofing layer by the embryo during the course of incubation—an event which occurs during the develop-

ment of the *Rhodnius* egg (Beament, 1948*b*). Indeed the inner membrane, which is secreted after 2–3 days of incubation, might possess the necessary waterproof properties. In order to resolve this question further evidence was sought regarding the permeability of the layers of the egg-shell both to water and to other substances.

TABLE 8. *The number of eggs, in batches of 10, that shrivelled in dry air at 25° C. after the following treatments: a, b, c, extraction in cold chloroform for 1 minute, 30 minutes, and 4 hours, respectively; d, dipping in alumina dust; e, rubbing with dust*

| Days of incubation | Treatment | No. of eggs shrivelled after | | |
|---|---|---|---|---|
| | | *30 min.* | *4 hrs.* | *24 hrs.* |
| 0–1 | a | 9 | 9 | 9 |
| | b | 10 | .. | .. |
| | c | 10 | .. | .. |
| | d | 2 | 2 | 10 |
| | e | 10 | .. | .. |
| 1–2 | a | 3 | 4 | 8 |
| | b | 7 | 10 | .. |
| | c | 10 | .. | .. |
| | d | 0 | 0 | 8 |
| | e | 8 | 10 | .. |
| 3–4 | a | 0 | 0 | 0 |
| | b | 8 | 10 | .. |
| | c | 10 | .. | .. |
| | d | 0 | 1 | 3 |
| | e | 3 | 6 | 9 |
| 6–7 | a | 0 | 0 | 1 |
| | b | 7 | 7 | 8 |
| | c | 10 | .. | .. |
| | d | 0 | 0 | 2 |
| | e | 0 | 2 | 8 |

*Permeability of the Shell Layer.* Unwaterproofed eggs laid during the previous 24 hours were exposed to solutions of many different dyes, solvents, oils, and salts, but it was soon apparent that particles of a very wide variety of molecular sizes, and having either hydrophilic or lipophilic affinities, were capable of passing rapidly into the yolk. Stains, such as basic fuchsin, penetrate the shell layer and stain it intensely; and it is also stained if the dye is injected into the egg. Large protein molecules will also penetrate the shell. If, for example, the egg is immersed in laked blood for 24 hours the colour of the contents deepens as oxyhaemoglobin enters the yolk. Now it is known that the normal brownish tint of the *Ornithodorus* egg is due to the presence of haemoglobin pigments (Wigglesworth, 1943). As the shells of ovarian eggs at the stage of yolk deposition are no less permeable than one-day eggs, there is clearly no obstacle to the absorbtion of these pigments through the shell

layer from the haemolymph. The large pore size might also facilitate the penetration of the relatively small wax molecules into the lipophilic shell layer.

The waterproofed egg in the first day of incubation is, by contrast, entirely impermeable to all staining solutions unless they are incorporated with vigorous wax solvents. After treating the egg with the wax emulsifier C09993 or cold chloroform for increasing periods, the permeability increases progressively and the staining properties of the shell layer are restored. At first the eggs swell osmotically in distilled water but fail to shrink in saturated sodium chloride, indicating that the shell is now permeable to small ions as well as to water. Extraction for 30 minutes in cold chloroform renders the shell permeable to sucrose but it still stains only in small and isolated patches. However, after extraction in boiling chloroform for 5 minutes the original permeability of the shell is completely restored and it then stains deeply and uniformly. These observations must imply that there is considerable affinity between the wax and the shell material.

*Permeability of the Inner Membrane.* Normal eggs incubated for 3 days at 25° C. (and therefore possessing a completed inner membrane) were extracted for 5 minutes in boiling chloroform. After this treatment the egg-shell as a whole was found to be permeable both to water and to monovalent ions, but, although the shell layer stained deeply with aqueous basic fuchsin, the dye no longer entered the yolk. This suggested that the inner membrane was permeable to water and salts but not to larger molecules.

The properties of the inner membrane may be tested more directly by carefully puncturing and stripping off the shell layer from the three-day waterproofed egg. The inner membrane, which is left entire as a delicate pellicle surrounding the yolk, is then found to be very permeable to water, as is shown by its swelling properties. Its outer surface, which is matt and seemingly homogeneous, is fairly readily wetted by water. On the other hand, if the shell layer is removed from an egg in the eighth day of incubation, the inner membrane is distinctly greasy and hydrofuge; and the egg swells much more slowly in distilled water.

Although this evidence does not entirely exclude the possibility that the inner membrane is impregnated from within by lipoid secreted by the embryo, it seems much more probable that the wax deposited on the outside of the egg gradually migrates through the shell layer as far as the inner membrane. Since the inner membrane is at first freely permeable to water, the diminishing influence of chloroform and abrasive dust on transpiration cannot be ascribed to the deposition of this layer. It is also noteworthy in this connexion that the effect of these agents gradually declines throughout the period of incubation—a result which might be expected from the slow infiltration of wax into the shell layer—whereas the inner membrane is laid down at a particular stage of incubation. These changes in the egg-shell during the incubation period are indicated diagrammatically in Text-fig. 12A, B.

If no secondary waterproofing layer is secreted after the onset of incubation, there should be no marked change in the critical temperature of the egg. The

'evaporation curves of batches of eggs incubated for 6 days at 25° C. is shown in Text-fig. 4. The critical temperature is approximately 44° C. and compares with a value of 43° C. for the one-day eggs. Nevertheless, the lower rate of water loss above the critical temperature, which is brought out clearly in this figure, is a very constant feature of the older eggs. It is possible that we are observing here a further effect of the impregnation of the shell layer with wax (see p. 330).

<center>DISCUSSION</center>

Our observations have shown that the tick egg is waterproofed by an external covering of wax which is secreted by the female tick after the shell layers are complete. This is the reverse of the condition found in *Rhodnius* and other insect eggs where the impermeable wax layers are laid down by the oocyte or embryo after the formation of the chorion and therefore lie inside it (Beament, 1946*b*, 1948*b*). From the superficial position of the wax layer it follows that tick eggs are very readily attacked by wax solvents, detergents, or abrasive dusts; whereas these agents often exert a remarkably small effect on the transpiration from many insect eggs.

An additional property of the waxy covering is to cause the eggs to adhere in a cluster—a function often assumed by the cement-secreting glands in insects. Among the Argasidae the wax layer responsible for the impermeability of the general integument is protected by an external covering of cement (Lees, 1947). Nevertheless, the manner in which the egg is waterproofed is obviously incompatible with the acquisition of a further protective layer. And our results show clearly that the waxes are in fact freely exposed on the surface of the egg.

In *I. ricinus* wax is applied to the outside of the egg in two stages. An incomplete layer, secreted probably by the lobed accessory glands, is first smeared over the egg during its passage down the vagina; and a further complete covering of wax is applied when the egg touches Géné's organ. On the other hand, there is no evidence that the egg of *O. moubata* is even partially waterproofed before it is laid: here Géné's organ is solely responsible for secreting the waterproofing layer. This difference may possibly be related to the size of the egg. In *Ixodes* (as indeed in all Ixodidae) the egg is relatively small and has an approximate volume and surface area of only 0·045 mm.³ and 0·61 mm.² respectively, as compared with values of 0·33 mm.³ and 2·3 mm.² for the *Ornithodorus* egg (Text-fig. 13A, F). Whereas the rate of water loss from the egg may be expected to vary according to the surface area, the amount of water that can be lost before desiccation becomes critical, and also the rate of shrinkage of the egg, will be determined by the volume. It is indeed an observed fact that unwaterproofed eggs of *Ixodes* (but not those of *Ornithodorus*) shrink so rapidly that there would clearly be some danger of excessive desiccation taking place during the manipulations of the egg by Géné's organ, were the first incomplete wax layer not already present.

Evidence, both of an observational and experimental nature, shows clearly that the function of Géné's organ is to transfer a waterproofing wax to the

surface of the egg. The gland is a proliferation of the general epidermis which is folded inwards from the cuticle in this region. This relationship provides a simple explanation of the fact—which at first sight appears as a striking example of parallel physiological adaptation—that the eggs of a given species of tick have the same order of resistance to desiccation as the parent species itself. The production of a waterproofing agent is a general property of the epidermis and Géné's organ can be regarded as a region specialized for waterproofing the egg. Nevertheless, there are important differences in the properties of the egg and cuticular waxes which can, in turn, be related to their specific functions (see below).

A consideration of the nature of the wax precursors raises some interesting questions which are worthy of further study by more exact chemical methods. Although it has proved impossible to isolate the wax itself from this situation, it is highly probable that the wax precursor is present in the watery contents of the horns of Géné's organ. Protein is also present, but the nature of its association with the wax is not clear. Certainly the wax is not dispersed as a coarse oil-in-water emulsion stabilized by protein (in a manner analogous to the dispersion by protein of the poly-isoprene aggregates in rubber latex). Against such an interpretation may be cited the absence of visible droplets in the horn contents, the insensitivity to changes in pH and to the presence of excess polyvalent ions, and the stability to heat. An alternative suggestion that the horns may contain emulsifying agents, other than protein, receives no support from the experimental results, for the secretion from the horns is found to be entirely devoid of emulsifying properties when tested against the wax deposits from the outer surface of Géné's organ. It is more likely that the wax is intimately associated, and probably chemically linked, with protein when it is secreted by the gland. Although the nature of such lipo-protein complexes is but imperfectly understood (Chargaff, 1944), the action of proteins in dispersing water-insoluble lipids, like cholesterol, is well known.

The stability of the wax-protein association is such that it is difficult to believe that the wax could be liberated by any purely physical process, for example, by ultrafiltration through non-living cuticle. And the cuticular wall of the horns is devoid both of pore canals and of any cytoplasmic lining. We have shown, however, that pore canals, penetrating the cuticle, are present around the base of the horns where the gland epidermis becomes applied to the inner surface of the endocuticle; and there is also a zone where the pore canals remain uncovered by the cement which forms an external covering over the basal part of the stalk. If, as appears probable, this is the site of wax secretion on the outer surface of Géné's organ, the horns must serve as reservoirs for containing the relatively large quantities of wax precursor needed for the rapid waterproofing of a batch of eggs. Such a function must also imply that the precursor secreted by the gland is first stored in the horns and is then again taken up by the gland-cells as required and passed through the cuticle by the cells which maintain connexion with pore canals. The form in which the wax is transported through the cuticle is, of course, unknown,

but the highly soluble wax-protein complex would appear to be a material very suitable for transport by the cell. At the cell surface, perhaps at the tip of the pore canals, the complex appears to be broken down and the wax in some way released. The protein moiety must then be retained by the cell, for, as we have seen, the wax deposits on the outside of Géné's organ are protein-free.

Since Géné's organ makes contact with only a small part of the total surface area of the shell during normal oviposition movements, the newly secreted wax must have the ability to spread and complete the waterproof layer over the egg. We have shown that although wax from the deposits on Géné's organ does possess definite powers of spreading on unwaterproofed eggs and, under very favourable circumstances, on prepared membranes, these powers are unexpectedly feeble. By comparison, for example, with the waterproofing grease from the cockroach cuticle (Beament, 1945), spreading of the egg waxes is slow and inefficient. The lipophilic properties of the shell material and the uniformly smooth surface of the shell layer are undoubtedly factors which favour spread. There is no reason to suppose, however, that the extension of the wax film is also assisted by the presence of specific spreading agents on the egg surface. Perhaps the wax undergoes further chemical changes after secretion, resulting in a partial loss of the spreading properties.

The comparison of the critical temperatures of tick cuticles and eggs also suggests points of interest in relation to the spread of waterproofing materials. In two species of *Ornithodorus* the critical temperatures of the egg, although comparatively high, are about 20° C. lower than are those for the cuticle of the parent species. These differences are correlated with the physical properties, the natural wax from the cuticle being hard and crystalline whereas that from the egg is soft and viscous. It would seem likely that the properties of the egg wax are largely dictated by the need for spreading over the surface of the shell. In general, it has been found that species of insects with high critical temperatures are waterproofed by hard apolar waxes (Wigglesworth, 1945; Beament, 1945). One might suggest from the knowledge at present available that a wax with good spreading powers is unlikely to have a high critical temperature; and that this property will be achieved only at the expense of some increase in permeability. Unlike the egg waxes, the cuticular lipoids have little need for mobility as spreading may be limited to the distance between neighbouring pore canals. Among Ixodidae there are comparatively slight differences only between critical temperatures of cuticle and egg in the same species. Nevertheless, preliminary observations suggest that the waxes concerned differ in their physical properties as widely as in the Argasidae; the egg waxes always appear to be more mobile.

A notable feature of the development of the tick egg is the fact that the shell is secreted not by follicle cells, but by the oocyte itself. The microscopic structure of the shell is comparatively simple. Only three layers are present when the egg is laid, the outer wax layer, a layer of granules with reducing properties, and the shell layer; a fourth layer, the inner membrane, which,

like the shell material, is composed of protein, is added after several days of incubation.

The unwaterproofed shell layer is remarkably permeable both to water and to large molecules with either lipophilic or hydrophilic affinities. Our experimental results point to the conclusion that the mobile wax gradually invades and impregnates the shell layer and in places reaches the inner membrane. The changes in the permeability of the egg around the critical temperature are probably related to these events. It has been suggested that increased transpiration through the insect cuticle at the critical temperature is associated with the destruction by thermal agitation of the parallel alinement of the wax crystallites at the protein-wax interface (Beament, 1945). If the shell layer is pictured as a loose protein meshwork, the wax will at first be entirely superficial and temperature will exert a correspondingly profound effect on transpiration. As the wax infiltrates into the shell layer and not only occupies the protein interfaces throughout the thickness of the layer, but also fills the intermolecular pores, the permeability to water may be expected to fall. This may be the explanation of the lower transpiration rate of 6-day eggs above the critical temperature. There is no evidence that a similar relationship is ever established between the cuticular waxes of ticks and the cuticulin-polyphenol substratum on which they are deposited. Here the waterproofing layer remains entirely superficial and therefore continues to be accessible to such agents as abrasive dusts (Lees, 1947).

SUMMARY

1. During the oviposition of ticks a glandular organ—the organ of Géné—is everted and touches the egg. If it is prevented from everting most of the eggs shrivel rapidly; few hatch even in a humid atmosphere.

2. The waterproofing properties of the normal egg are conferred by a superficial coating of wax, $0·5-2·0\ \mu$ in thickness. In *Ornithodorus moubata* the wax is secreted and applied solely by Géné's organ. In *Ixodes ricinus* waterproofing takes place in two stages: an incomplete covering of wax, probably secreted by the lobed accessory glands, is first smeared over the egg during its passage down the vagina; waterproofing is then completed by a further application of wax from Géné's organ after the egg has been laid. Owing to its superficial position on the egg the wax layer is readily attacked by solvents and emulsifiers.

3. The morphology of Géné's organ in *O. moubata* is described. The gland is a proliferation of the epidermis which lies detached from the cuticle. Its secretion, a watery refractile liquid containing the wax precursor, accumulates between the gland and the cuticle in two horn-like extensions. The wax is probably secreted through pore canals distributed over a narrow zone of cuticle below the horns; the cement covering-layer of the epicuticle does not extend to this zone.

4. The transparent, heat-stable material isolated from the horns of Géné's organ is regarded as the wax precursor. Solubility in water is probably con-

ferred by chemical linkage with protein. The precursor is taken up from the horns, where it is stored, and is presumably broken down within the gland cells. The wax is then secreted through the pore canals while the protein moiety is retained by the cell.

5. The critical temperatures of the eggs of Ixodidae range from 35° C. in *I. ricinus* to 44° C. in *Hyalomma savignyi*; only slightly higher critical temperatures were recorded for Argasidae (45° C. in *O. moubata*). Eggs with lower critical temperatures are more susceptible to desiccation. The susceptibility of the eggs of a given species is of the same order as that of the parent species; but whereas in Ixodidae the critical temperatures of the egg and the cuticle of the female tick are approximately the same, in Argasidae the critical temperatures of the cuticle are much higher (62° C. in *O. moubata*). These differences are related to the physical properties of the waxes. The cuticular wax in *O. moubata* is hard and crystalline (m.p. 65° C.), whereas the egg wax is soft and viscous (m.p. 50–54° C.).

6. The natural wax from Géné's organ has definite powers of spreading on the surface of the egg and so completing the waterproofing layer.

7. The material extracted with boiling chloroform from egg-shells or from nymphal cuticles separates spontaneously into two fractions, a hard white wax (*c.* 85 per cent. by weight) and a soft yellow grease (*c.* 15 per cent.). The properties of these two lipoids differ conspicuously from those of the natural wax. Attempts to deposit the extracted materials on membranes in the form of a waterproofing layer were unsuccessful.

8. Ovulation is described in *O. moubata*. The shell of the tick egg is secreted by the oocyte itself and not by follicle cells. Three layers can be distinguished in the 24-hour egg: (i) an outer wax layer; (ii) an incomplete layer of granules which reduce ammoniacal silver nitrate; (iii) a shell layer. A fourth layer, the inner membrane (iv), is secreted by the oocyte after incubation for 2–3 days.

9. Both the shell layer and the inner membrane are composed of resistant, elastic protein and are devoid of chitin. The shell layer of the unwaterproofed egg is highly permeable to water and to large molecules with either hydrophilic or lipophilic affinities. The inner membrane is at first freely permeable to water and to inorganic ions. During the course of incubation the wax gradually migrates into the shell material and may reach the inner membrane. As this occurs, the effectiveness of abrasive dusts and of chloroform in promoting increased transpiration through the shell is notably reduced.

## REFERENCES

ADAM, N. K., 1945. Bull. ent. Res., **36**, 269.
BEAMENT, J. W. L., 1945. J. exp. Biol., **21**, 115.
—— 1946a. Quart. J. micr. Sci., **87**, 393.
—— 1946b. Proc. Roy. Soc. B, **133**, 407.
—— 1948a. Bull. ent. Res. (in the press).
—— 1948b. Ibid. (in the press).
BERTKAU, P., 1881. Verh. naturh. Ver. preuss. Rheinl., **38**, 146.
CHARGAFF, E., 1944. *Advances in Protein Chemistry*, vol. 1, pp. 1–24. New York (Academic Press Inc.).
CHRISTOPHERS, S. R., 1906. Sci. Mem. med. sanit. Dep. India, no. 23.
DOUGLAS, J. R., 1943. Univ. Calif. Publ. Ent., **7**, 207.
GÉNÉ, J., 1848. Mem. d. R. Acad. Sci. Torino, **9**, 751.
LAING, J., 1935. Quart. J. micr. Sci., **77**, 497.
LEES, A. D., 1947. J. exp. Biol., **23**, 379.
LEWKOWITSCH, J. I., and WARBURTON, G. H., 1921. *Chemical Technology of Oils, Fats and Waxes*, vol. 1, pp. 317–49. London (Macmillan).
LOUNSBURY, C. P., 1900. Ent. News, **11**, 336.
MANTON, S. M., 1938. Phil. Trans. B, **228**, 421.
NORDENSKIÖLD, E., 1909. Zool. Anz., **35**, 30.
NUTTALL, G. H. F., 1908. J. Inst. Publ. Health, **16**, 385.
RAMSAY, J. A., 1935. J. exp. Biol., **12**, 373.
ROBINSON, L. E., and DAVIDSON, J., 1914. Parasitology, **6**, 382.
SAMSON, K., 1909. Z. wiss. Zool., **93**, 185.
SMITH, C. N., 1945. Ann. ent. Soc. Amer., **38**, 223.
WHELER, E. G., 1906. J. agric. Sci., **1**, 400.
WIGGLESWORTH, V. B., 1943. Proc. Roy. Soc. B, **131**, 313.
—— 1945. J. exp. Biol., **21**, 97.
—— 1947. Proc. Roy. Soc. B, **134**, 163.

## EXPLANATION OF PLATE I

Fig. 1. *O. moubata*. Female tick, with Géné's organ everted, clipped between glass slides. ×7.

Fig. 2. *O. moubata*. Sagittal section through the centre of an everted Géné's organ to show continuity of epidermis (indicated by arrows) with the gland. ×50.

Fig. 3. Géné's organ in *O. moubata*. Gland epithelium showing secretory droplet. ×210.

Fig. 4. The same, showing relation of gland to the thin cuticle of the horns. ×210.

Fig. 5. Whole mount of Géné's organ in *O. moubata*. The tick with the organ everted was immersed for 30 minutes in ammoniacal silver nitrate. Note absence of staining. ×20.

Fig. 6. A similar organ extracted for 30 minutes in cold chloroform and stained with ammoniacal silver. Note intense staining over the horns where cement is absent. ×20.

Fig. 7. *O. moubata*. Cuticle of the horns heavily stained with silver after chloroform extraction. There are no pore canals in the thin unstained endocuticle. Section photographed in water. ×430.

Figs. 8, 9. *O. moubata*. Sections of the cuticle from the zone between silver-staining and non-silver-staining areas of Géné's organ. The thickness of the cuticle is indicated by an ink line, pore canals in the endocuticle and silver-staining areas of the epicuticle by arrows. ×430.

Fig. 10. *I. ricinus*. Section through one lobe of the gland of Géné's organ. Honeycomb border indicated by arrow. Iron haematoxylin. ×210.

Fig. 11. *I. ricinus*. Portion of lobed accessory gland, to show similarity with gland epithelium of Géné's organ. Iron haematoxylin. ×210.

Fig. 12. *I. ricinus*. Longitudinal section of vaginal cells and circular muscles. Iron haematoxylin. ×210.

Fig. 13. Transverse section of tubular accessory gland. Iron haematoxylin. ×210.

Figs. 14, 15. *O. moubata*. Surface view of shell fragments from two unwaterproofed eggs treated with 5 per cent. ammoniacal silver nitrate. ×210.
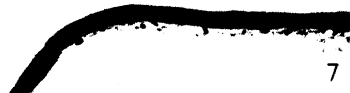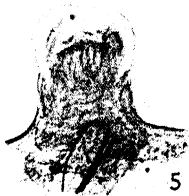
# A Study of the Spheroid System of Sympathetic Neurones with Special Reference to the Problem of Neurosecretion

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**With two Plates**

## INTRODUCTION

IN the year 1891 Camillo Golgi of Pavia in a short article entitled 'Sur la structure des cellules nerveuses' announced the discovery of his now-famous 'Apparato Reticulo Interno'. He states:

L'aspect caractéristique de cet appareil réticulaire interne peut provenir de la forme prédominante en ruban, des fils, du mode de se diviser, de s'anastomoser et du cours de ceux-ci (spécialement dans les grandes cellules on observe un cours nettement tortueux), de la présence dans cet appareil de minces plaquettes ou de petits disques arrondis et transparents dans le centre, qui forment comme des joints nodaux du réseau, et enfin de la couleur spéciale, jaunâtre, que prennent les fils par effet de la réaction.'

This celebrated discovery as so often happens was made by chance, the new 'apparatus' appearing at the behest of the 'black reaction' which occurs when nervous tissues previously soaked in a solution of dichromate are placed in silver nitrate.

Since the time of Golgi the homologues of this nerve-cell 'apparatus' have been studied in many kinds of cells by a great number of workers (Hirsch, 1939, in his recent monograph lists over 2,000 references to this topic).

Because of the great diversity of form the apparatus may take when studied by the classical techniques of silver and osmium impregnation in diverse cells, or even in identical cells within a tissue, authors frequently fail to reach agreement on the precise identification of the apparatus. Confusion has been the inevitable result and in some quarters a degree of scepticism prevails even to this day concerning the existence of this organella—the sceptics ascribing the appearance in the histological preparations to artifacts of technique; a view strengthened by the difficulty of adequately studying the apparatus in the living cell.

In an attempt to resolve this morass of conflicting opinion, Baker (1944) set out to find whether the fully developed Golgi element of diverse cells could be shown to conform to a single structural plan. His researches led him to conclude that in the nerve-cell this element consists not of elaborate peri-

nuclear networks but of separate discrete or 'dispersed systems' lying in the cytoplasm. These systems consist of a lipoid sheath or pellicle enclosing a more or less fluid core which can be stained during the life of the cell by neutral red chloride. Further, he indicated that this binary system of bodies may occur widely in other tissues and there represent the true Golgi complement of the cells.

Baker preparations of nerve-cells are indeed objects of great interest and considerable speculation to the neuro-histologist. The appearances are novel and cannot easily be reconciled with the classical Golgi networks of the metallic impregnation techniques, so much so that some workers would rather incline to the view that a completely new cytoplasmic organella has been demonstrated in the nerve-cell.

Consequently it has been thought advisable to carry out a further investigation of this problem and to try to establish the identity of the Baker bodies on the one hand with structures visible within the living cell, while on the other with the classical appearances of the silver and osmium techniques. The interesting new technique of phase-contrast microscopy has been utilized as far as possible in this investigation.

## METHODS

The freshly excised superior mesenteric ganglia of mice were examined in a few drops of 0·7 per cent. sodium chloride containing 0·2 per cent. of 10 per cent. anhydrous calcium chloride; the fluid was warmed to 37° C. in an incubator. Both transmitted and phase-contrast illumination were employed in this study. The details of the phase-contrast microscope used will be described in a forthcoming article by Kempson, Thomas, and Baker.

Both supravital and intravital staining were carried out with neutral red chloride B.D.H., methylene blue B.D.H., and Janus black B.D.H. dissolved in the required concentrations in 0·7 per cent. sodium chloride solution containing 0·2 per cent. of 10 per cent. calcium chloride. For intravital staining 2 c.c. of a 1 per cent. solution of neutral red chloride in distilled water was injected intraperitoneally following a preliminary ether induction. Coal-gas was used for killing the animals.

For fixed material the following techniques were employed:

1. Baker (1944) sudan black technique for the demonstration of the Golgi apparatus in frozen sections.
2. The variant of the above technique applicable to paraffin sections as described on p. 340.
3. Mann–Kopsch technique.
4. The Azan stain of Heidenhain and Masson's trichrome stain following Zenker Formol fixation and post-chroming at 37° C. for 3 days in a saturated solution of potassium dichromate in distilled water.
5. Feulgen reaction.
6. Celestin blue–pyronin reaction of Sanders (1946).

*The Living Cell*

The superior mesenteric ganglion of the mouse was found to be a very suitable tissue for high-power microscopic observations of the living sympathetic neurone. The ganglion was rapidly dissected with the aid of a Greenough binocular microscope and placed in a capsule of warmed 0·7 per cent. sodium chloride containing 0·2 per cent. of 10 per cent. anhydrous calcium chloride. Because of the small size of this ganglion and its general transparency further teasing of the material is unnecessary. If the unsupported coverglass is placed upon the tissue the resulting pressure is sufficient adequately to flatten the cells and thus allow a clear microscopic image of the cytological details of the surface cells to be obtained.

The cytoplasm of the ganglion cell is seen to contain a variable number of small roundish bodies more or less evenly distributed throughout. These bodies vary in size from single small spherical homogeneous granules to larger compound objects which appear to be made of two parts, a somewhat yellowish spherical core to which are attached around the periphery smaller darker grains or crescentic caps. The largest bodies are frequently somewhat irregular in contour and are closely similar to, although smaller than, the 'mulberry spheroids' described in *Helix* neurones (Thomas, 1948). In this respect the general similarity of structure of the living vertebrate sympathetic neurone to the living invertebrate cerebral neurone is indeed very striking. Further, if one only examined preparations fixed in Formol-saline or Bouin's fluid stained with haematoxylin and eosin the impression would be gained that these cells possess a clear homogeneous cytoplasm whereas in point of fact the exact opposite is true: the living cell is crowded with a large assortment of diverse granular bodies scattered throughout its cytoplasm.

*Vital staining with Neutral Red and Janus Black*

*Neutral red chloride* 1–10,000 in saline is a very valuable dye for clearly revealing these intracellular bodies. In a short while after its application to the tissue the spherical bodies absorb the dye and become tinged a red colour throughout.

It must be stressed that the neutral red solution merely colours pre-existing bodies which can be readily seen within the unstained cell. There is no suggestion, under the conditions of the staining experiment outlined in the 'Methods' section of this paper, of the formation of unspecific neutral red segregation droplets within the cells. Exactly the same is true for *Helix* neurones.

As the colour begins to fade from the vitally stained tissue owing to the reduction of the dye to its leucobase the duplex bodies are particularly well differentiated into their component parts—a bright staining pellicle to a more or less faintly tinged core. Apparently the pellicle, which in some cases appears as a complete skin to the core and in others as attached granules or

crescentic caps, has a far greater capacity once stained to hold the dye and to resist its eventual reduction.

*Methylene blue* was similarly applied both vitally and *intra vitam* as this dye has been advocated by Worley (1943) as a stain of high specificity for the 'externum' of the Golgi systems of a number of cells studied by him and his co-workers. In my hands his methods gave results which were exactly comparable to those given by neutral red. In some cases the differentiation of the duplex bodies is even more striking. Text-fig. 1 is an accurate camera-lucida drawing of a cell stained with methylene blue. It will be noticed that the larger systems may measure $2\mu–3\mu$ in diameter. The smallest methylene
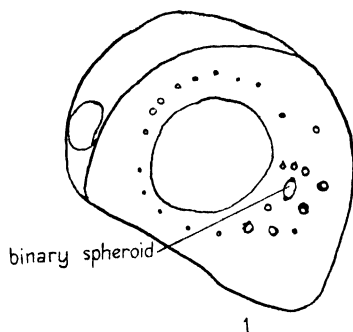


TEXT-FIG. 1. A mouse sympathetic neurone stained vitally with methylene blue. The spheroids exhibit attached blue-staining granules and crescents. Camera-lucida drawing.

blue-stained granules are apparently homogeneous or at least are too small for accurate description as binary systems. Similar bodies have been seen by Hirsch (1940) in *Ascaris* and interpreted by him as remnants of the Golgi apparatus (Golgi 'Rest') which serve as a reserve of material apparently capable of development into binary systems as circumstances require.

*Janus black* possesses the property of staining very specifically the mitochondria to the exclusion of the spheroidal bodies. This technique although difficult will often give good results if repeated trials are made. The mouse ganglia were again chosen for their small size and transparency which allows observation of intact cells undisturbed in relation to their surroundings. Suitably stained cells reveal the presence of numerous delicate filaments or moniliform chains tinged a greenish-black colour. In the most successful preparations the mitochondria curiously appear to be situated immediately beneath the cell membrane, an appearance probably attributable to the fact that these cells have been but slightly penetrated by the dye and only the peripherally situated mitochondria are stained and clearly differentiated against an as yet clear central mass of cytoplasm containing the remaining unstained mitochondria. In the same way stained cells fail to reveal more deeply situated mitochondria as the very delicate filaments do not concentrate

enough dye to 'show through' a thick layer of slightly diffusely stained cytoplasm. Text-fig. 2 is a camera-lucida drawing of a mouse ganglion cell stained vitally with Janus black and showing numerous mitochondrial filaments. This drawing should be compared with Pl. I, fig. 4, which shows exactly similar objects in the fixed cell after post-osmification in the Mann–Kopsch technique.

Janus green B, one of the most popular dyes for showing mitochondria in living cells, was found unsuitable for mouse cells. As always with Janus



mitochondrion

10μ

2
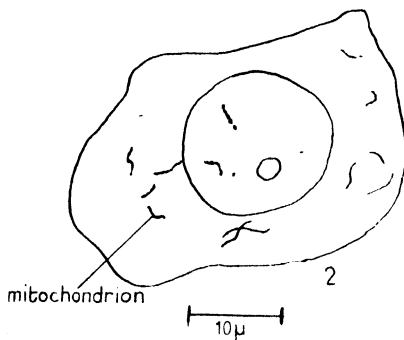
TEXT-FIG. 2. A mouse sympathetic neurone stained vitally with Janus black. The filamentous mitochondria are stained a greenish black. Camera-lucida drawing.

staining, dyes of different origins and at varying concentrations should always be applied in order to obtain the most satisfactory results.

*Fixed and Stained Material*

*Reactions to osmium tetroxide.* If small pieces of mouse ganglia are suspended in saline as a hanging drop on a cavity slide, and a small drop of 2 per cent. osmium tetroxide solution placed in the cavity but arranged so as not to touch the hanging drop (Text-fig. 3), the effects of osmic vapour can be studied on the spheroid bodies while fixation of the living cell is taking place. After a period of a few hours these bodies assume a brownish colour.

If tissues are immersed in a simple solution of osmium tetroxide and left overnight in the incubator the spheroids can be totally blackened.

In order to study what part if any these osmiophil bodies may play in the subsequent changes known to occur in the cell during prolonged osmification, it was decided to carry a number of pieces of tissue through one of the classical osmium techniques for the demonstration of the Golgi apparatus, and to withdraw and examine some of the pieces at regular intervals during the stated period (usually 6–10 days) of impregnation. The Mann–Kopsch technique was chosen for this experiment, pieces of ganglion being fixed for 2 hours in Mann's mercuric-chloride-osmium solution and transferred after

washing to 2 per cent. osmium tetroxide and placed in the 37° C. incubator. At 12-hourly periods a piece was removed, washed, dehydrated, embedded, and cut at $7\mu$.

The discrete spheroids are plainly visible in the first batch of sections after a total of 12 hours' immersion. Very frequently in suitable cells one can find the appearance shown in Pl. I, fig. 3. The smallest granules are homogeneously blackened, but in the case of the larger ones only their outer coats are impregnated, revealing a more or less colourless centre. Frequently this osmiophil pellicle shows local thickening which appears as caps or crescents applied to the spheroids. Sometimes linear bands occur around the core.

After 48 hours, nearly all the spheroids are totally blackened, only the largest bodies retaining the clear central area. As the degree of osmification
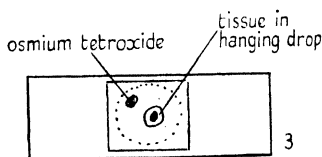


TEXT-FIG 3.

of the cells varies considerably within any one section, material left for 3 days will usually show all these stages from incomplete to complete blackening of the spheroids in the same section.

Within 24 hours in addition to the spheroid bodies a second osmiophil element in the form of delicate filamentous threads becomes visible in the cells (Pl. I, fig. 4). At first these threads are but faintly tinged a light greyish colour, but as osmification proceeds their colour darkens and the objects appear thicker, longer, and more tortuous. Adjacent filaments soon appear to coalesce to form long wavy bands which frequently appear to fuse with the already blackened spheroid bodies which then become incorporated as so many nodular swellings upon the bands (Pl. I, fig. 5). Lateral anastomoses of these nodular bands occur as more and more reduced osmium is superimposed in a non-specific manner upon this scaffolding originally formed by two separate discrete elements, the filamentous threads and spheroid bodies. In this way an apparently unrelated and new structure is built up within the cell which at first sight bears no relationship whatsoever to any object or objects visible within the living cell. The life-like appearance of the cell seen after short periods of osmification (12–36 hours) is altogether lost as the Mann–Kopsch process proceeds with its snowball-like deposition of osmium throughout the cell. That there is a general non-specific deposition of osmium particles throughout the cell seems certain from the generalized gradual darkening of the cytoplasm as a whole as osmification proceeds. Eventually the whole cell can be blackened throughout and made quite opaque if tissues are left for a long time (10–20 days) in osmium solution at raised temperatures

(37° C.). It seems reasonable to assume that much of this non-specific deposition of osmium particles would take place first about a centre of already impregnated intracellular objects, and that this centre would itself be transformed in a haphazard manner into a thickened, distorted, and unrecognizable object as superimposed layers of metallic particles are built up upon it.

After 6 days the great majority of the cells exhibit the classical Golgi appearance (Pl. I, fig. 6). The cell shows a generalized blackening but more especially around the anastomotic network of impregnated strands. In some cases the originally single spheroid bodies are completely lost within the 'black reaction'. In other cells they can still be discerned either incorporated within the meshwork itself or lying between the strands.

## The Application of Sudan Black to the Fixed Cell

Baker (1944) introduced a new method for the study of the Golgi element of cells. He applied the substance sudan black, which strongly colours the lipoidal external pellicle of the system so as to differentiate these bodies in frozen sections. Rabbit anterior mesenteric ganglia were chosen for study as they are considerably larger than in the mouse and their frozen sections are large enough to afford easy handling with the technique. The formal calcium sudan method applied to these cells reveals the presence of a number of small clear vacuoles scattered throughout the cytoplasm. Each vacuole is bounded by a delicate sudanophil skin which is characteristically concentrated and heaped up on one side, giving to the whole body a signet-ring appearance. Sympathetic neurones prepared by this method have a very novel appearance which cannot at first sight be reconciled with any of the classical cytological pictures of the neurone. One may almost be excused in interpreting the vacuole with its lipoid skin as a new nerve-cell organella.

The vacuoles vary fairly considerably in size within any one cell and correspond in size and general distribution with the spherical bodies observable within the living cell and stainable vitally with neutral red. The neutral red staining caps or crescents in the pellicle of the living duplex system very closely resemble the appearances of the 'heaped-up' sudanophil rims to the vacuoles.

As in the case with the smallest osmiophil bodies a clear vacuole cannot always be resolved in the centre of the body. Such objects appear as very small sudanophil grains just resolvable with the microscope and would correspond to the 'Praesubstanz' or 'Rest' of Hirsch.

It must be stressed, however, that the formal-calcium sudan black technique as applied to frozen sections fails to reveal within the sympathetic cell any obvious deposits of lipoidal granules, as may frequently be found in resting gland cells or such as the lipochrome deposits of invertebrate neurones which are always strongly sudanophil.

Deposits of black 'melanin' granules are sometimes described in sympathetic cells. I have never found them in rabbit ganglia.

Attempts were made to develop a technique to preserve these sudanophil vacuoles in paraffin sections. The following method proved a useful one for this purpose:

1. Fixation in acetic-osmic-bichromate of Bensley's or Helly's fluid followed by post-chroming at 37° C. with a saturated aqueous solution of potassium dichromate. (The dichromate salts conserve the lipines by converting them into alcohol-insoluble substances which remain sudanophil after embedding.)

2. Wash 4–6 hours at the tap.

3. Dehydrate and embed in paraffin. Cut and mount sections on slides with albumen solution in the usual manner.

4. Take the section down through xylene and the alcohols, to 70 per cent. alcohol—leave 3 minutes.

5. Place in saturated sudan black in 70 per cent. alcohol 7–10 minutes.

6. Quickly rinse in 50 per cent. alcohol to remove surface stain particles.

7. Rinse in water, counterstain in carmalum, and mount in Farrants's medium.

With this technique the cores of the spherical bodies appear as faintly yellowish-brown masses and not clear vacuoles as in the frozen sections. Closely applied to the core are a number of strongly sudanophil granules or crescents. Sometimes there is a more or less complete sudanophil pellicle to the core but the more general appearance is of attached granules, whereas in the frozen sections crescents predominate. The largest spheroid bodies often have a large number of dense black granules almost completely covering their surfaces.

Careful focusing is necessary in order to see the yellowish-brown core within the cluster of granules.

Pl. I, figs. 1 and 2, are a photomicrograph and a drawing of a cell exhibiting a number of spheroids with their black sudanophil pellicle. The core of the two largest spheroids can be distinctly seen surrounded by numerous small granules. Such large mulberry forms occur frequently and appear to have a somewhat irregular outline and uneven contour suggestive of their being possible aggregates of smaller spheroids.

Some cells show large numbers of small sudanophil granules often aggregated into clumps at one pole of the cell. Usually a few typical spheroid systems can be seen within this clump. It may be argued that some of these small granules could be Rests from which new systems might be formed, but as a similar appearance does not occur in control material after formal-calcium fixation it seems more likely that they are precipitates of cytoplasmic materials invisible in the living material. Since these sudanophil granules only occur following the use of fixatives containing protein precipitants, the assumption is that they must constitute in life a lipoprotein complex which is split by the fixative into its constituent parts, the fatty element being thereby unmasked and preserved in the sections by the action of the dichromate. Pl. II, fig. 1,

illustrates a clump of these granules following Helly's fixation and stained with sudan black.

## Reactions of the Fixed Cells to the Trichrome Techniques

In view of the interesting researches of Scharrer and others upon the intraneuronal 'secretion' granules, the Azan and Masson trichrome techniques were applied to paraffin sections of Helly-fixed rabbit sympathetic ganglia.

Morphologically two types of stainable granules can be seen in the cells by the application of these techniques, viz.:

(1) Perfectly spherical discrete globules showing variations in size from very small to medium-sized granules staining red with either azocarmine or the acid fuchsine of the Masson technique.

(2) Much larger irregular granules having a mulberry or compound appearance. Sometimes a large granule may be surrounded by an almost complete ring of much smaller granules in very close contact with it. Sometimes as many as four or five large mulberry granules may occur together within one cell (Pl. II, fig. 3). These bodies similarly stain with azocarmine or acid fuchsine.

(3) Clumps of ill-defined brownish-yellow granules occur in some cells. They are usually gathered together characteristically in one pole of the cell (Pl. II, fig. 6). These granules only occur following fixation with protein precipitants when combined with dichromate and are of a fatty nature as they stain with sudan black. They are discussed above and will not be referred to in this section. They do not appear in tissues fixed in lipoid solvent fixatives such as Carnoy's solution.

The azocarminophil and fuchsinophil granules are of regular occurrence throughout the tissue. These granules can also be stained well with Heidenhain's iron haematoxylin. They give a negative reaction to Sakaguchi's arginine reaction but a mild positive xanthroproteic. They are negative to both the Feulgen and celestin blue pyronin reaction (see Sanders, 1946).

With the latter technique the Nissl substance is particularly well shown and does not bear any positional relationship to the granules described above. One of the most striking and curious features concerning these granules in the fixed cells and a very frequently occurring phenomenon is illustrated in Pl. II, figs. 4, 5, 6. Here an azocarmine-staining granule appears stuck upon the nuclear membrane, which itself is strikingly modified for a considerable distance on each side of the zone of contact. With the Azan technique this thickened zone of the nuclear membrane is usually coloured purplish-blue in contrast to the brilliant red granule. The photographs give but a poor impression in monotone of the striking appearance.

In some instances the granule appears to be partly within the nucleus itself, suggesting to the observer that the granule was either passing in or out from cytoplasm to nucleus or vice versa when the cell was fixed (Pl. II, fig. 6; the granule referred to is the black object shaped like a thick plus sign, lying on the nuclear membrane).

Occasionally similarly staining but smaller granules can be seen lying within the nucleus itself, where they can be differentiated from the single large nucleolus by their smaller size and tinctorial properties.

The nucleolus not infrequently loses its somewhat woolly spherical form and is modified into a somewhat fan-shaped structure which approaches and becomes contiguous with the nuclear membrane from within (Pl. II, fig. 7). This phenomenon is not associated with any modifications of the nuclear membrane as is the case when a cytoplasmic granule is applied to it.

It was felt desirable to correlate if possible the granules seen in the fixed and stained cell with the spheroid bodies and granules visible in the living cell and stainable in a differential manner by the vital dyes, neutral red and methylene blue, and by the sudan black method on frozen sections.

The technique described above, whereby sudan black is applied to paraffin sections followed by mounting in Farrants's medium, serves as a valuable means of bringing about this correlation. The following steps were carried out:

1. Fix material in Helly's fluid and post-chrome for 3 days at 37° C. in a saturated solution of potassium dichromate. Section by the paraffin method and colour with sudan black. Mount in Farrants's medium.

2. Photograph suitable cells and record their position with the stage verniers (Pl. II, fig. 1).

3. Remove the coverglass and leave in running water to remove the Farrants's medium (5 minutes).

4. Extract the sudan black by placing the slide in 70 per cent. alcohol for 5 minutes.

5. Place in water 1 minute.

6. Stain the section according to the Azan technique. Mount in balsam and re-photograph and compare the same cells (Pl. II, fig. 2).

In this way it can be shown that the azocarmine-staining granules possess a sudanophil sheath or coat which can at once be identified with that of the spheroid binary systems present in the living or vitally stained cell and with the vacuoles with lipoid skins demonstrated in frozen sections.

In other words, this staining experiment proves that the granules, which are so strikingly shown with the trichrome stains, form within the core to the spheroid systems possessing lipoidal sheaths, as described previously.

### DISCUSSION

#### *The True Form of the Golgi Apparatus*

As a natural development from the pioneering researches of Parat (1928) within recent years, much of the confusion has been resolved concerning the cellular organella usually described as the Golgi apparatus. From the important researches of Hirsch (1939), Worley (1943, 1944), and Baker (1944), many new facts have been brought to light on this vexatious problem, and it is

becoming increasingly clear that this important zone of the cell consists of a number of discrete binary systems consisting of a lipoidal pellicle enclosing a more or less fluid core. This core constitutes the 'vacuome' of Parat and it is thought forms the site of production of most endocellular secretion antecedents, i.e. Golgi products.

The methods of attack on this problem have altered materially within recent years, and a new school of cytologists has developed which no longer bases conclusions on the structure of the Golgi system solely from the observation of its appearance in fixed material impregnated by the older classical silver and osmium methods.

The phase-contrast microscope has provided biologists with a tool particularly applicable to the study of the living cell. In the author's hands this instrument fails to reveal any structure within living nerve-cells, either invertebrate or vertebrate, that can be reconciled with the classical dictyosomes (Gatenby) or Golgi networks of the metallic impregnation technique, and he feels that another blow, perhaps a fatal one, has been dealt to earlier doctrines of workers such as Beams (1931), Hirschler (1918), Gatenby (1924), and others who have in a wide variety of studies upheld the classical teachings of Golgi (1891), Veratti (1898), and Cajal (1915).

In this study living vertebrate sympathetic neurones (of mouse and rabbit) have been shown to contain a number of discrete bodies which range in size from small grains through simple spheroids to larger bodies possessing an irregular contour.

The following facts have been established about these bodies:

1. They are easily visible in the living cell, appearing slightly yellowish with transmitted light.

2. They are stainable vitally with neutral red or methylene blue. As the colour fades from the preparations the bodies are differentiated into two parts, a pellicle and core.

3. They are clearly differentiated by Janus black or Janus green B in the living cell from the filaments or moniliform chains of the mitochondria.

4. They are tinged a brown colour with osmic vapour or totally blackened if immersed in a solution of osmium tetroxide.

5. They appear as vacuoles with lipoidal skins showing local thickening in frozen sections fixed in formal-calcium solution and when coloured with sudan black.

In the past it has been generally considered that valuable information on the Golgi could only be gleaned from tissues that had remained in osmium or silver solutions for a 'specified' time (with the osmium methods usually 6–10 days). At the conclusion of the procedure, should the tissues fail to reveal the established appearances it was usually assumed that the failure was due to 'over-' or 'under-' impregnation. In short, the older Golgi technique could be described largely as a cytological ritual to which the would-be-

successful technician must conform. It is surprising that more attempts have not been made to study the changes within the cell from the living state to the 'completed impregnation'.

It has long been acknowledged that the osmium Golgi techniques are superior in reliability to the silver methods. Consequently in this research the Mann–Kopsch technique was chosen for such an analytical study.

The experiments with this technique show very clearly that the most life-like picture of the cell is produced by very short periods of osmification, namely, 12–18 hours. Then the binary nature of the spheres is clearly differentiated as the pellicle is much more osmiophil than the core, but as osmification proceeds this appearance is lost and they become homogeneously blackened.

The filamentous mitochondria are similarly osmiophil and when they first appear in the Mann–Kopsch technique their size and configuration closely match their appearance in the living cell stained with Janus black. Apparently the mitochondria form the material basis for the subsequent progressive deposition of further particles of reduced osmium, for as the osmification proceeds the individual filaments are thickened and lengthened until finally adjacent threads appear to anastomose and link up one with the other in an irregular fashion.

The spheroid bodies in the main lose their general characteristics as a dispersed system of individual bodies and become either incorporated in the tangle of thickened threads or are lost in the concomitant non-specific deposition of osmium particles which has taken place throughout the cytoplasm but more especially about the original mitochondrial framework. In this way at the end of 6–10 days these preparations reveal a picture at once identifiable with the classical Golgi network.

These results conform to the view that the classical Golgi network of the sympathetic neurone is an appearance produced in the cell in an artificial manner, namely, by a non-specific deposition of metallic particles about and upon two distinct categories of pre-existing intracellular organellae, the spheroid systems and the mitochondria.

I associate my results with the views of Hirsch, Worley, and Baker, from their studies of a number of diverse cells, and conclude that in the sympathetic neurones the Golgi substance consists of a number of dispersed binary systems possessing lipoidal chromophil capsules to a neutral red staining core —the vacuome of Parat.

It is indeed fascinating to read the original description of the apparatus from Golgi's pen in his article of 1898 (see introduction to this paper). It is very curious that his 'little rounded discs' which he describes as interposed upon the network like nodes, and 'transparent in the centre', should have for so long escaped the attention of the scores of subsequent investigators, whose attention seems to have been mainly directed to conserving the 'network' in their preparations to the detriment of the 'discs with transparent centres'—a component readily accessible in the living cell.

*The Homology of the Golgi Element in Invertebrate and Vertebrate Neurones with the Golgi Elements of the Germinal Cells*

The rod-shaped lepidosomes of the reproductive cells of Gastropods have long been claimed as the homologue of the Golgi apparatus of the neurone (Hirschler, 1918; Gatenby, 1924; and others). To support this claim the dictyosomes and archoplasm of the invertebrate neurone have been put forward as an intermediate link or transitional form of the apparatus. According to this view the individual rodlets of the dictyosome correspond to dispersed pieces of the vertebrate Golgi network, whereas the attached archoplasm represents a remnant of the condensed cytoplasm of the invertebrate Nebenkern. These views can be briefly summarized by the following schematic diagram:
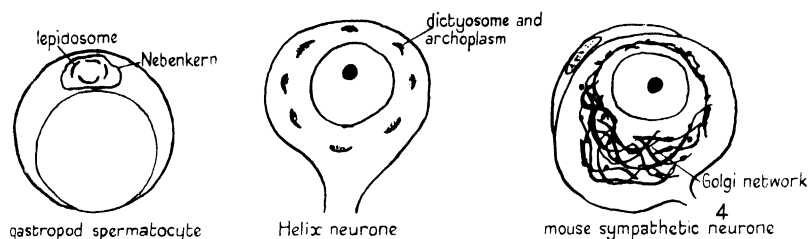


Text-fig. 4. Schematic diagram of the homology between (*a*) Gastropod spermatocyte, (*b*) Gastropod nerve-cell, (*c*) Mammalian sympathetic nerve-cell (according to Gatenby and others).

On the other hand, Parat in 1928 demonstrated that the vacuoles present in the Nebenkern and first seen by Platner (1885) in the living cell could be stained vitally with neutral red. Parat then launched his vacuome theory, which suggests that the vacuoles are, in fact, the true Golgi complement of the spermatocyte. The somewhat enigmatic lepidosomes he interprets as modified mitochondria (the 'chondriome actif'). Baker (1944) has recently reinvestigated this problem and has shown that the vacuome of Parat has an external skin or coat consisting of lipines, and he identifies the binary system so formed as the Golgi material.

The author (Thomas, 1947) has previously made a detailed study of the invertebrate (molluscan) neurone with the phase-contrast microscope, controlling the observations with the newer techniques of Baker (sudan black) and Worley (methylene blue), and has put forward facts which strongly suggest that the earlier views identifying the dictyosome as the Golgi material are mistaken. Instead the cell has been shown to possess a well-marked system of spheroids possessing lipoidal capsules and stainable vitally with neutral red and methylene blue. These systems are unmistakably homologous to the spheroid bodies of the vertebrate sympathetic cell described in this paper.

Although it seems that further work is required on the problem of the

Nebenkern of gastropod spermatocytes, it is now reasonably certain that a strictly homologous series of binary spheroidal bodies exists in invertebrate and vertebrate neurones and that these bodies are, in fact, the true Golgi material of the neurone. Further, these Golgi systems would appear homologous to the vacuoles of Platner of the invertebrate spermatocyte together with the lipoidal coat to the vacuole.

The following diagram expresses this new interpretation of the homology.
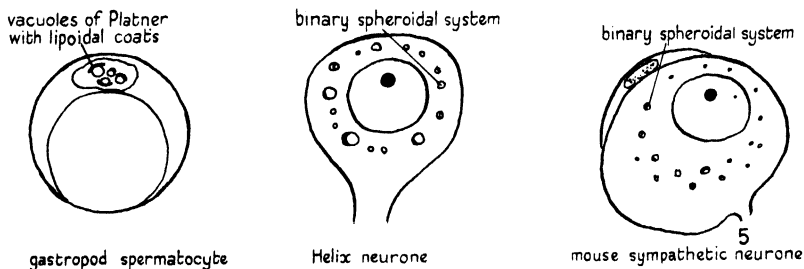


TEXT-FIG. 5. Schematic diagram of the author's conception of the homology between (a) Gastropod spermatocyte, (b) Gastropod nerve-cell, (c) Mammalian sympathetic nerve-cell.

It must be stressed that these views preclude any recognition whatsoever of a classical Golgi network as a performed living structure within the neurone. Instead this network is claimed as an artificial distortion of two separate categories of real structures—the mitochondria and the spheroid bodies.

The spheroid bodies which can be clearly followed through from the living cell to the fixed and impregnated preparation are the sole contributors to the Golgi complement of both the vertebrate sympathetic neurone and the invertebrate cerebral ganglion cells.

It would appear better to abandon the word 'Golgi apparatus' or 'Golgi material'. If a dispersed system of binary spheroids can be shown to be of general occurrence in cells, as may well be the case, a new cell nomenclature is clearly required. However, for the time being we must use for convenience such expressions as 'true Golgi complement', while bearing in mind that the object named by Golgi in nerve-cells consisted not of the binary spheroids only, but of the mitochondria and of irregular depositions of silver as well.

*Intraneuronal Granules and the Problem of Neurosecretion*

Within recent years a number of workers (Scharrer, 1940; Palay, 1943; *et al.*) have put forward the thesis that nerve-cells in certain situations can in addition to their purely conductive capacity elaborate and discharge 'neurohormones' into the blood-stream. Recently Lennette and Scharrer (1946), studying the sympathetic nerve-cells of the monkey, have demonstrated the presence of granules, remaining in the cytoplasm after celloidin embedding and hence thought to be protein in nature, which may be interpreted as a secretion antecedent. As they failed to produce evidence of a secretion cycle

they deemed that their material was not favourable for a cytological study of the problem.

In my paraffin-embedded material granules with precisely the same morphogenic and tinctorial properties occur widely throughout the tissue. Further, it has been shown that these granules are intimately associated with the Golgi spheroids. The smallest granules appear to be formed within the lipoidal pellicle of a single Golgi system for it can be shown that they are at first completely covered with a sudanophil skin. This finding is in exact accord with the observations of Hirsch and others who have shown that in exocrine gland cells, e.g. the pancreas, the secretion antecedents always make their first appearance in this situation and later emerge from the pellicle as free and independent zymogen granules.

The large irregular 'mulberry' granules appear to be formed by the concerted efforts of a group of Golgi Rests. In this case the individual Rests remain as independent small sudanophil bodies and it is possible that each contributes its quota of Golgi product to form collectively the 'mulberry' granule. Groups of small Rests are frequently found in the Helly-fixed material either without or associated with granules of varying size. Text-fig. 6 illustrates in a schematic way these two possible mechanisms for the formation of Golgi product in the neurone:
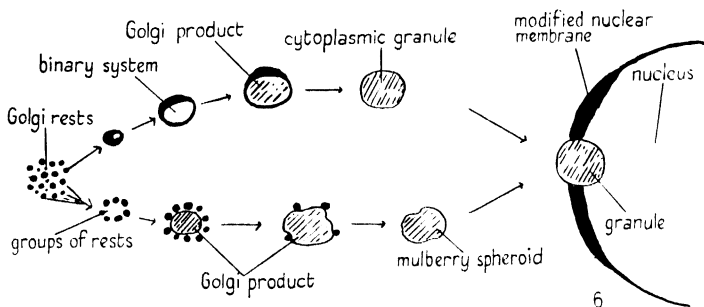


TEXT-FIG. 6. Schematic diagram to show the two possible mechanisms for the production of Golgi product within sympathetic neurones. The product is transported to the nucleus.

When the Golgi material is in contact with the nucleus the preparations resemble some of the stages of nucleolar emission reported in ova during yolk formation in *Saccocirrus* (Gatenby, 1922) and *Diemyctylus* (Saguchi, 1932). I wish to thank Professor J. Brönte Gatenby for allowing me to examine some of his preparations of *Saccocirrus* and to compare them with my own material. Nucleolar emission has also been described by Carlier (1902) in liver, gastric epithelium, and salivary gland cells of rat, cat, and newt, and by Page May and Walker (1908) in nerve-cells of mammals. After a careful study of the latter paper I have concluded that the phenomena met with in my sections are altogether different. Nuclear protrusions as shown in Page May and Walker's figs. 11 and 12 do not occur in my material;

instead the granules of Golgi material appear to apply themselves to the undeformed nuclear membrane and to spread out in a fan-shaped manner over its surface, contributing to the characteristic thickened area of nuclear membrane in the zone of contact (Plate II, figs. 4, 5, 6).

As the granules survive treatment with the alcohols and xylene during the embedding processes and further do not themselves colour with sudan black, it is likely that they are of a protein nature. Further, they give a weak xanthroproteic reaction.

Their reaction with the nucleus is a very curious one. The whole appearance suggests, although it cannot be proved, that this material originally formed within the Golgi systems migrates to the nucleus and is absorbed by it.

It might be argued that the converse may well be the case and that the material has a nuclear origin and is extruded from the nucleus into the cytoplasm, as Caspersson (1939) and others have shown is the case for particles of ribose nucleic acid.

The following points can be made in favour of the former views:

1. The material, whatever its exact chemical nature may be, gives a negative Feulgen reaction and is not stained by pyronin, so it seems certain that we are not dealing with either desoxy-ribose or ribose nucleic acid.

2. The nerve-cell has been shown to contain a dispersed system of Golgi spheroids exactly comparable to the systems that Hirsch and others have shown to exist in normal glandular cells, wherein all secretion antecedents originate within the chromophobe portion of the Golgi system and are finally set free within the cytoplasm. In the vertebrate sympathetic cell, as is the case with the molluscan neurone, we can demonstrate all the stages typical of a secretory cycle of Golgi origin. In *Helix* neurones the Golgi product tends to be fatty (the granules of Legendre) whereas in the sympathetic neurone of mammals protein products predominate.

In brief, a secretion cycle can be demonstrated for the cytoplasmic origin of these granules quite apart from any suggestion of their being of nuclear origin.

The Golgi products described in this study are clearly identical with the 'secretion antecedents' claimed by Scharrer and others who subscribe to the neurosecretion hypothesis. The observations concerning the nuclear absorption of these granules may well cast some light on the problem of neurosecretion in general.

It must be admitted that, however interesting these observations and conjectors may be, the central problem of the functional significance of the material of the neurone must remain unsolved until direct experimental evidence can be brought to bear on the problem.

It is the author's hope that these newer concepts of the cytology of the neurone originally proposed by Baker and expanded in this study will bear fruit in the hands of workers generally concerned with problems of nervous physiology and pathology.

In conclusion I wish to thank Dr. J. R. Baker for his ever-ready help and encouragement throughout this study and Prof. A. C. Hardy, F.R.S., for his help in preparing the text. Also to Mr. Humphrey Leach of the Physiology Dept., Oxford, and Prof. J. Brönte Gatenby, Dublin, for helpful criticism and advice.

## SUMMARY

1. Evidence is put forward in support of the view that the Golgi complement of the vertebrate sympathetic neurone consists of a dispersed system of spherical bodies. These bodies can be studied in living cells and with vital dyes and each consists of a neutral red staining core (the vacuome of Parat) enveloped in a lipoidal sheath.

2. The classical Golgi body is shown to be an artifact produced within the cell. With the osmium techniques the spheroid bodies together with the mitochondria form a framework which serves as a centre for a non-specific deposition of metallic particles.

3. The Golgi spheroids exhibit a secretion cycle with the formation of a granular product. The granules are identified with the 'neurosecretion' granules of Scharrer.

4. These granules appear to be transported to the nucleus of the cell and there to be absorbed. This observed interrelationship between nucleus and Golgi product is discussed.

## DESCRIPTION OF PLATES

### PLATE I

FIG. 1. A photomicrograph of a sympathetic neurone fixed in Helly's fluid, post-chromed and coloured with sudan black. Two large spheroids with lipoidal pellicles contain mulberry granules (unstained). Some smaller systems are also shown.

FIG. 2. Camera-lucida drawing of the same cell.

FIG. 3. Camera-lucida drawing of a sympathetic neurone 12 hours after the commencement of the Mann–Kopsch technique. Only the pellicles of the spheroid systems are blackened.

FIG. 4. Camera-lucida drawing of a sympathetic neurone 36 hours after the commencement of the Mann–Kopsch technique. The spheroids are mostly blackened throughout; in addition numerous filamentous mitochondria have become blackened by the osmium.

FIG. 5. Camera-lucida drawing of a sympathetic neurone 3 days after the commencement of the Mann–Kopsch technique. The mitochondria are thickened and anastomose. Some of the spheroids have become incorporated on the strands so formed. The cell cytoplasm shows some general blackening.

FIG. 6. Camera-lucida drawing of a sympathetic neurone 6 days after the commencement of the Mann–Kopsch technique. The classical Golgi network is now formed. Individual spheroids are still recognizable. The cell cytoplasm shows a general non-specific deposit of osmium particles more especially around the network.

### PLATE II

All photomicrographs were taken with the technique of tube length extension described by Dr. J. R. Baker in a special article on photomicrography in the *Journal of the Royal Microscopical Society*, vol. 62, p. 112. A Reichert 2-mm. fluorite immersion lens and Watson Holos eyepiece 14 × were employed for Figs. 1, 2, 3, 4, and 5; for Fig. 6 a Zeiss 2-mm. apochromat with 10 × compensating ocular, and for Fig. 7 a Zeiss 1·5-mm. apochromat with 10 × compensating ocular.

FIG. 1. Two sympathetic neurones coloured with sudan black following Helly's fixation with post-chroming. Each cell contains one large spheroid system with sudanophil pellicle A and B.

FIG. 2. The same cells after removal of the sudan and restaining with the Azan stain. The cores of the two spheroids A and B contain azocarmine-staining granules.

FIG. 3. Large 'mulberry' granules in a sympathetic neurone of rabbit. Helly fixation post-chromed and stained with the Azan stain.

FIGS. 4, 5, 6. Sympathetic neurone of rabbit with azocarmine-staining granule applied to the nucleus. Helly fixation post-chromed. Azan stain.
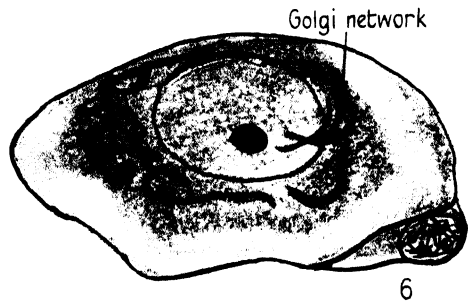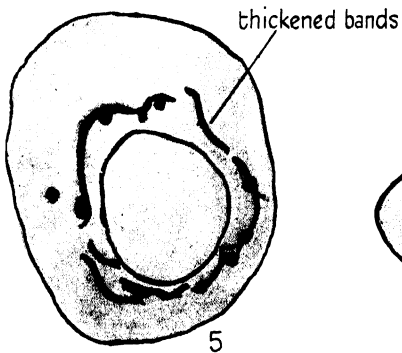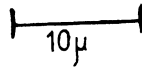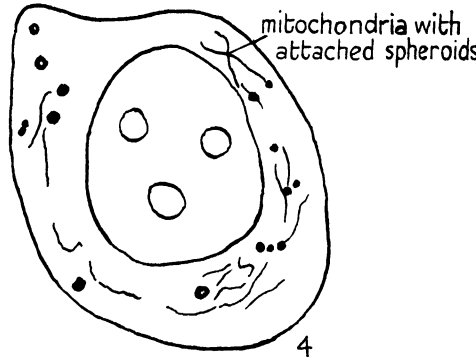
FIG. 7. A sympathetic neurone of rabbit with two nuclei. One azocarmine-staining granule in the cytoplasm. The nucleolus of the right-hand nucleus appears applied to the nuclear membrane from within. (A similar appearance to the 'Anlagerung' of Bargmann, 1948.) Helly fixation, post-chromed. Azan stain.

## REFERENCES

BAKER, J. R., 1944. Quart. J. micr. Sci., **85,** 1.

BARGMANN, R., 1948. (Personal communication.)

BEAMS, H. W., 1931. Anat. Rec., **49,** 309.

CAJAL, R. y., 1915. Trab. del Lab. de Investig. Biol., **12, 127.**

CARLIER, E. W., 1902. Proc. Scottish micr. Soc., **5,** 82.

CASPERSSON, T., 1939. Arch. exp. Zellforsch., **22,** 650.

GATENBY, J. B., 1922. Quart. J. micr. Sci., **66, 1.**

—— 1924. Scient. Proc. R. Dublin Soc., **17, 275.**

GOLGI, C., 1891. Arch. Ital. de Biol., **30,** 62.

HIRSCH, G. C., 1939. Form- und Stoffwechsel der Golgi-Körper, Protoplasma-Monographien, B. 18. Berlin (Borntraeger).

—— 1940. Protoplasma, **35,** 280.

HIRSCHLER, J., 1918. Arch. mikr. Anat., Bd. 91, Abt. 1.

LENNETTE, E. H., and SCHARRER, E., 1946. Anat. Rec., **94,** 85.

PAGE MAY, W., and WALKER, C. E., 1908. Quart J. exp. Physiol., **1,** 203.

PALAY, S. L., 1943. J. comp. Neurol., **79,** 247.

PARAT, M., 1928. Arch. Anat. micr., **24,** 73.

PLATNER, G., 1885. Arch. mikr. Anat., **25,** 564.

SAGUCHI, S., 1932. Zytologische Studien, Heft 5, Kanazawa.

SANDERS, F. K., 1946. Quart. J. micr. Sci., **87, 203.**

SCHARRER, E., and SCHARRER, B., 1940. Res. Publ. Ass. nerv. ment. Dis., **20,** 170.

THOMAS, O. L., 1948. Quart. J. micr. Sci., **88,** 445.

VERATTI, E., 1898. Anat. Anz. **15,** 190.

WORLEY, L. G., 1943. Proc. Nat. Acad. Sci., **29, 228.**

—— 1944. J. Morph., **75,** 261.

Mulberry spheroid
with sudanophil
granules attached

small
binary systems

2

10 μ

binary
system

3

mitochondria with
attached spheroids

4

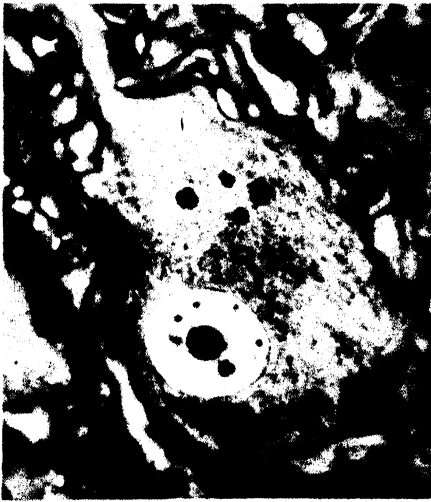10 μ

thickened bands

5

Golgi network

6

O. L. THOMAS—PLATE I
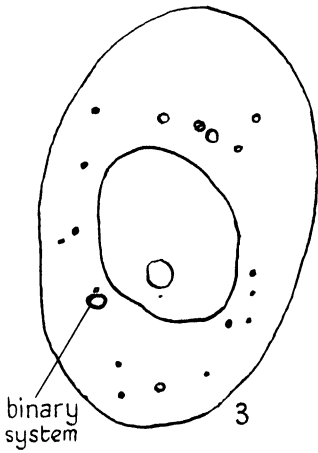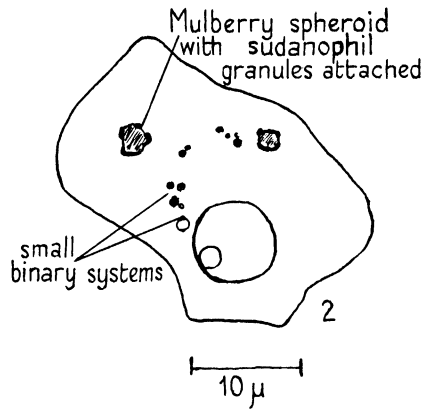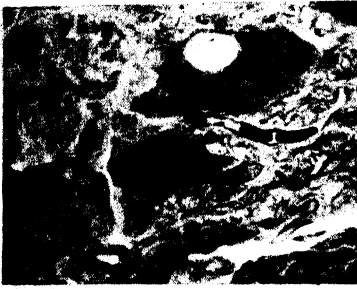
# A Simple Method for Phase-Contrast Microscopy

BY

D. A. KEMPSON, O. L. THOMAS,[1] AND JOHN R. BAKER

*(From the Department of Zoology and Comparative Anatomy, Oxford)*

THE method described in this paper enables anyone to use phase-contrast microscopy without waiting to obtain special objectives or condensers.

The phase-contrast method is particularly valuable in the examination of living cells with high powers. The most perfect lenses for studying living cells in body-fluids or saline solutions are water-immersion lenses, since these are specially corrected for the use of aqueous media below the coverslip. For this reason we shall describe the adjustment of a microscope to give the phase-contrast effect when a Zeiss 2·5 mm. apochromatic water-immersion lens of N.A. 1·25 is used. Although others will not use precisely this lens (and will in many cases probably use oil-immersion lenses instead), we think it useful to give actual measurements of the apparatus we describe, to serve as a guide.

The first thing to do is to prepare a phase-plate, which, in our method, will lie close *behind* (*above*) the back focal plane of the objective. Our method is not applicable to objectives carrying a phase-plate *in* the back focal plane. Obtain from a firm of lens-manufacturers a circle of glass, 1 mm. thick, of the same diameter as the back lens of the objective, and with the two sides optically plane and exactly parallel with one another. Our circle is 5·5 mm. in diameter. This plate will be made into a phase-plate, but it is necessary first to construct a special cell to hold it in position, close behind the back lens of the objective. In our apparatus the distance between the back lens and the phase-plate is 0·77 mm. The cell that maintains the plate in this position in the objective serves also to hold it while the annulus is being fashioned on its surface, as will be described below.

The cell in our apparatus (see Text-fig. 1) has the general appearance of a funnel-stop used in dark-ground illumination, but is carefully designed in such a way that it does not reduce the N.A. of the objective. It is essentially a hollow cylinder, holding the phase-plate at its lower end and secured to the objective at its upper end. The manner in which the back lens of the objective is mounted may necessitate making the walls of the cell at the phase-plate end very thin; but this is a desirable feature, for these walls by being slit can function as a spring chuck or collet, thus permitting the changing of plates.

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Most objectives have a screw-in stop in the form of a ring at the back end of the mount, and if the cell is made so as to occupy the whole of the space inside, with proper clearance for the phase-plate, the ring provides a convenient method for holding it in. Slots must be cut at several places at the mouth or phase-plate end of the cell, thus forming spring jaws. A seating must be turned out inside the mouth, at a depth of approximately half the thickness of the plate. This shouldered seating ensures that the plate is held square to the
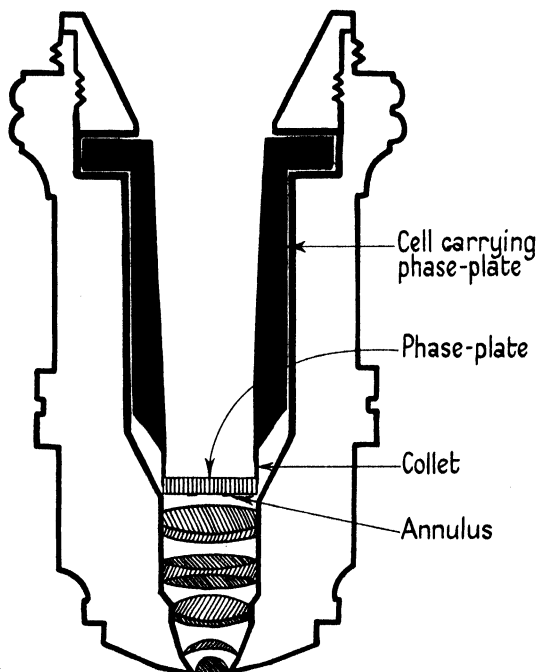


TEXT-FIG. 1. Longitudinal section of an immersion objective carrying a phase-plate. (The thickness of the annulus on the phase-plate has been greatly exaggerated, because it would otherwise be invisible in side-view.)

optical axis. As it is desirable to be able to change phase-plates conveniently and without causing damage, the jaws should be made very slightly bell-mouthed so as to avoid chipping the edges of the glass, which is very prone to fracture if pressure is applied at one point.

The use of another simpler cell will make the insertion of the plate into the holding cell much easier and will avoid the risk of chipped edges caused by handling with forceps. This merely consists of a small block of metal with a hole turned out and shouldered, in which the plate can rest freely to a depth of less than half its thickness. Place the plate into this recess with the

bloomed surface downwards and load into the proper cell by inverting the latter over it and gently pressing until seated squarely in position.

To make the annulus, a uniform layer of 'bloom' must first be deposited on the whole of one side of the plate. Send the glass plate to a firm that provides a 'blooming' service for photographic lenses (e.g. Messrs. Pullin Optical Co., Ltd., Phoenix Works, Great West Road, Brentford, Middlesex). Since the thickness of bloom is not always exactly the same, it is a good plan to have several plates bloomed, and to find which one works best in practice with particular objects.

Our phase-plates are of the kind called 'A—' by Bennett (1946): that is to say, the annulus is raised above the surface of the glass, and darkened by a slight deposit of opaque material above the bloom. To make this type of phase-plate, the bloom must be completely removed everywhere except in the annulus itself. Remount it in the cell. A very simple form of lathe can be adapted from the ordinary turn-table used in mounting microscopical slides. There must be no free play in the bearing of the revolving disc, which should be well lubricated. In the centre of the disc, fix a simple chuck by means of sealing-wax or even plasticine. This chuck is easily made from $\frac{1}{8}$-in. walled brass tubing, $\frac{5}{8}$ in. long and $1\frac{3}{8}$ in. in diameter, with three 4-B.A. screws tapped through to a common centre, equally spaced near one end of the tube. Mount this on the disc with the centring screws uppermost. Mark one of the screws and also the cell, so that the latter may be taken out and replaced in the chuck without much re-centring. By trial and error the phase-plate in its cell must be accurately centred in the chuck by adjustment of the screws, so that no lateral movement is perceptible as the disc is revolved. This operation and the turning off of the coating described later, require the use of a dissecting binocular, preferably of the long arm type, though it might be possible to manage with a lens fixed to a stand. Final centring requires very careful manipulation of the screws and may be helped by holding a needle (not by hand) close to the edge of the plate as it revolves. Incorrect centring causes the plate to move to and from the needle.

Originally the coating was scraped off by holding in the hand a very fine chisel-pointed needle, which was applied to the surface as it revolved. A simple device was latterly used, however, which gave precision-control of this operation and is strongly recommended. One requires a piece of wood to fit on top of the hand-rest of the slide-mounting turn-table, of such thickness that it is not higher than the phase-plate on the disc. Cut from thin sheet tin an L-piece $\frac{1}{2}$ in. wide, with arms 9 in. and $2\frac{1}{2}$ in. long. Bore a hole through the angle of the L. Pass a nail or screw through this hole and thus attach the L-piece to the wood in such a way that it swivels without play. The long arm of the L must point to the left, and the short one forwards, towards the middle of the turn-table. To make the scraping tool, grind a needle on an oil-stone to a fine chisel-point and fix it with plasticine to the end of the short arm, pointing it downwards at an angle of approximately 45°. Move and bend the short arm of the L-piece so that the scraper is about $\frac{1}{8}$ in. above the phase-

plate, thus allowing the forefinger to press it in contact with the surface of the plate; the spring tension lifts it off when pressure is released. The disc should be rotated anticlockwise and the scraping action commenced at the nine o'clock position. The left hand controls the long arm and thus slowly feeds the scraper across the surface of the phase-plate, while the forefinger of the right hand applies the pressure. The long lever effect permits precision control with comparative ease of operation.

Under the binoculars the scraped-off coating is plainly visible as a fine powder, making it quite easy to watch the process. With occasional wiping with a very soft brush, any part of the coating not properly removed can be seen at once. To scrape off the centre portion, begin at the centre of the plate, moving the scraper towards the nine o'clock position until the desired width of annulus is left.

Our annulus is 2·58 mm. in outer diameter and 1·52 mm. in inner diameter; it follows that the annulus is 0·53 mm. wide.

Needless to say, finger-marks are ruinous to results, and it is advisable to polish the back (unbloomed) surface of the plate thoroughly before it is placed in the cell.

In order to balance the direct light coming through the annulus with that of the diffracted light, carbon must be deposited on the annulus to reduce its transmission. By using a small flame, such as a cigarette lighter, with some xylene or benzene in the fuel, the plate can be smoked gradually to the desired density. Avoid overheating by occasional cooling to prevent the coating becoming temporarily soft. The density of the carbon of our best annulus has not yet been measured properly, but as a guide, it is between 1 and 1·5 photographic density, which is the equivalent of transmission of 10 to 3 per cent. The cell is taken out of the chuck, so that the rate of deposition of carbon can be watched by holding to the light. Replace in the chuck and centre accurately as before, remembering that the carbon is removed by the slightest touch. The carbon must now be removed from the clear glass, leaving it on the annulus only. Repeat the scraping technique to do this, but replace the needle with clean smooth-textured paper, cut to a fine tapering point. Examine the point carefully and remove projecting cellulose fibres. Recut the point if necessary, as the fibres are quite uncontrollable and will tend to remove carbon beyond the limit required. The removed carbon tends to build up into isolated heaps, which may be gently blown away with a pipette if care is taken that they do not touch the carbon on the annulus by being blown across its surface. The entire surface may be wiped clean from carbon with old clean linen if smoking has to be repeated.

The phase-plate, still held in its cell, is now to be placed in the objective. The side of the plate carrying the annulus will be downwards (that is, towards the back lens of the objective), as shown in Text-fig. 1. The whole optical system (except the eyepiece) is shown diagrammatically in Text-fig. 2.

A microscope-board is required to hold the lamp, illuminating-annulus, and microscope in correct alinement. Obtain a suitable piece of wood about

100 cm. long and 25 cm. wide, and make sockets at one end of it into which the feet of the microscope will fit, in such a way that it will always be held in exactly the same position. Incline the microscope at a comfortable angle, and make arrangements whereby it can be inclined at exactly this angle whenever it is desired to use the phase-contrast method.

The primary source of light must be rather intense. A 6-volt filament-bulb of the motor-car headlight type is suitable. It must be contained in a suitable housing, in which a focusing bull's eye must be incorporated. Screw the lamp-stand into the far end of the board from the microscope in such a position that perpendiculars dropped from the bulb and from the centre of the bull's eye would strike the board at the same distance from its long edge as would a perpendicular dropped from any part of the optical axis of the microscope. Now incline the lamp-housing and focus the bull's eye so that the image of the filament is projected on to the centre of the mirror of the microscope. The lamp-housing must now be clamped firmly, as its position should never be changed again; but it will be necessary to change the focus of the bull's-eye, without changing the direction of its central beam.

Two modifications of the light must now be made, the one for use in ordinary microscopy, the other for phase-contrast. For the former, a sheet of finely ground glass about 8 cm. square is suitable. It must be held in a retort stand provided with a rectangular base in such a way that when this base is pushed up against the side of the microscope-board, the centre of the ground glass plate lies on the line joining the centre of the bull's eye to the centre of the mirror, and the plane of the ground glass is at right angles to this line. The ground glass should be about 20 cm. from the mirror. The microscope is now ready for ordinary use.

For phase-contrast microscopy a second modification of the light is required —a *bright annulus*. Prepare it as follows. Take a piece of black paper about 8 cm. square, and cut out a circle 39 mm. in diameter. Stick the outer piece of black paper to a square sheet of glass of the same size. Now trim down the circle of black paper to a diameter of 27·5 mm., and stick it on to the same sheet of glass in such a way as to leave an annulus approximately 5·8 mm. wide between the two. It is a good plan to put four dots of Indian ink on the glass in the annulus, N., S., E., and W., as these will help in focusing the condenser at a later stage. Now take a square of tracing-paper, of the same size as the sheet of glass, damp it all over with medicinal paraffin, and apply it to the side of the glass that does not carry the black paper, carefully avoiding the inclusion of any air-bubbles. Fix this secondary source of light in a retort stand and place it in exactly the same position as that in which the ground glass stood before. The side of the glass carrying the tracing-paper should face towards the microscope. A drop of medicinal paraffin should be applied to the tracing-paper occasionally, if there is any tendency for it to dry up. (We have found tracing-paper damped with paraffin preferable to ground glass for this particular purpose.)

Everything is now ready for use. A well-corrected condenser, of medium

power, such as the Watson 'Parachromatic' (0·29 in. focus) should be used. Place some living cells in saline on a slide, cover, and place the slide on the microscope. Using the ground glass as the secondary source of light, focus a low-power objective on the cells. Now focus the condenser of the microscope in such a way that a pencil held against the ground glass is seen in focus at the same time as the cells. Replace the low-power objective by the water-immersion carrying the phase-plate, and focus it carefully on a cell. Now move the slide slightly, till there is no cell in the field of view. Remove the draw-tube from the microscope and screw a 3-in. objective into the bottom of it.
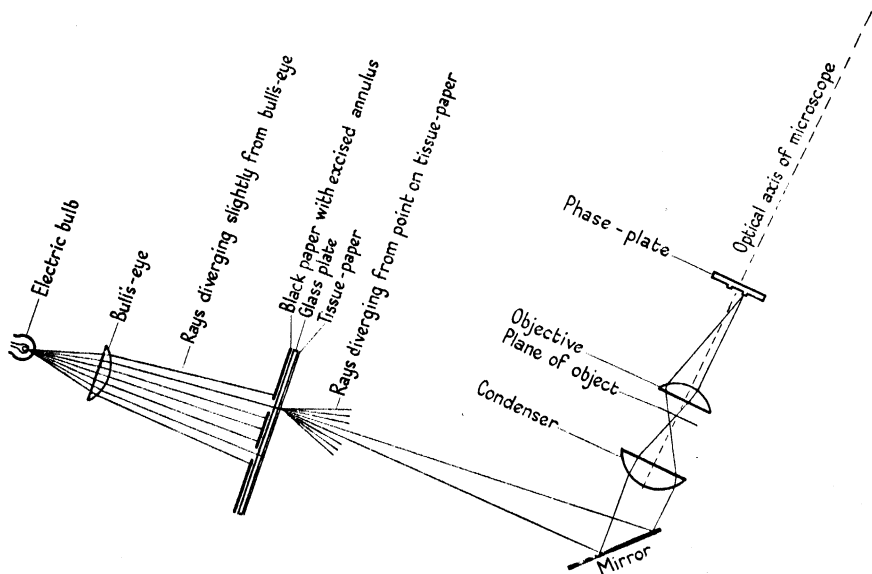


TEXT-FIG. 2. Diagram showing the optical arrangement described in the text. Two rays are followed from a point in the illuminating annulus to a point in the annulus on the phase-plate. (The eyepiece is not represented.)

Replace it in such a position that the annulus of the phase-plate is sharply focused. Be careful not to change the focus of the microscope.

Now take away the retort-stand carrying the ground glass and replace it with that carrying the annulus. Focus the bull's eye, if necessary, so that this annulus is brightly illuminated. (The bright circle cast by the lamp should be slightly larger in diameter than the annulus.) Look through the microscope and focus the condenser very slowly downwards. When a certain position has been reached, the bright annulus will be seen in focus at the same time as the phase-plate annulus. This will happen when the image of the bright annulus thrown by the condenser of the microscope lies in the plane that constitutes the focus that is conjugate to the plane of the phase-plate placed on the other side of the objective. The condenser is now in the 'low' position

recently described by one of us ('low-condenser illumination' (Baker, 1948)).

The object is to make the two annuli coincide. This is achieved (1) by adjusting the mirror; (2) by moving the bright annulus nearer to or farther from the mirror along the line joining the mirror to the bull's eye; and (3) by making corresponding movements of the condenser so as to keep the bright annulus in focus. The position is correct when the bright annulus coincides with the phase-plate annulus but is not quite wide enough to cover the whole of it, so that the edges of the phase-plate annulus are just seen outside and inside the image of the bright annulus. It is a very great advantage to use a stainless steel mirror instead of a glass one, so as to avoid double reflexions.

It is worth mentioning that exact focusing of the image of the illuminating annulus on the phase-plate annulus appears not to be necessary. Just as good results seem to be given if it is slightly out of focus, provided that all the direct (unscattered) light passes through the phase-plate annulus.

Now remove the 3-in. objective, replace the draw-tube, and move the slide until the cells come into view. 'Negative' phase-contrast has now been achieved: the field will appear grey, and objects of high refractive index bright. Nuclei will thus appear brighter than cytoplasm. Mitochondria and any bacteria will appear particularly bright.

When a satisfactory arrangement has been achieved, make marks on the microscope-board to enable you to bring the retort-stand (and hence the bright annulus) quickly into its correct position. With the phase-plate annulus described on pp. 353–4, and with a Watson 'Parachromatic' condenser, we find it necessary to place the bright annulus 20 cm. from the mirror.

It is a convenience to have a resistance attached to the microscope board in such a position that one may alter the intensity of the light without moving the eye from the microscope.

The method of illumination adopted bears a resemblance to that used by Burch and Stock (1942), but these authors used a straight slit instead of an annulus, and the light passed directly through it instead of being rediffused as in our system.

### SUMMARY

A method of phase-contrast microscopy is described, not involving the use of special objectives or condensers. A method for making the phase-plate carrying a raised annulus is described. A large annular source of light is

focused by the condenser of the microscope in a plane slightly *below* the object. The phase-plate is placed in the conjugate focus of this plane, just above the back lens of the objective.

## REFERENCES

BAKER, J. R., 1948. Quart. J. micr. Sci., **89,** 233.
BENNETT, A. H., 1946. Sci. Monthly, **63,** 191.
BURCH, C. R., and STOCK, J. P. P., 1942. J. sci. Instr., **19,** 71.

# Nervous Structure of the Spinal Cord of the Young Larval Brook-Lamprey

### BY

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**With two Plates**

IT is intended to describe here the spinal cord of recently hatched ammo-coetes of *Lampetra planeri* (Bloch). Particular attention will be given to those neurons which may be involved in the somatic sensori-motor arc. It is hoped to base upon this description a subsequent account of the embryo and young ammocoete, which will deal with the successive stages of nervous structure and their relation to the successive patterns of behaviour.

The neurology of the ammocoete, or pride, should be of particular interest, because great importance attaches to all aspects of the morphology of this animal in discussions of the phylogeny of vertebrates. For although the gnathostome vertebrates are not regarded as derived from some palaeozoic ammocoete, the morphology of the ammocoete certainly resembles that of the prototype from which higher vertebrates may be said to have evolved. This point of view is supported by the accounts of the general morphology of the nervous system of the young stages, and is borne out by the detailed neurology of the brain in the older stages and in the adults.

With very minor exceptions, the description of the nervous system of the younger stages has hitherto been made without the use of metallic impregna-tion or any other method of staining nervous tissue specifically, except in animals of above 4 cm. in length, that is in animals more than 1 year old (cf. Hardisty, 1944). But knowledge of the very youngest stages is urgently required; for the nervous organization of these could be compared with that found in amphibian embryos: and it is by a study of that organization in the embryos of the urodele that Coghill (1914, 1929, &c.) and Youngstrom (1940) have erected a far-reaching system of correlation between the successive structural patterns of the neurons during development and the stages of development of behaviour.

It is important to know how far Coghill's and Youngstrom's **conclusions are** applicable to other classes of vertebrates. Similar investigations **to those of** Coghill have been performed very effectively upon placentals (Barron, 1941). Upon fishes similar lines of research have been less successful. Descriptions of nervous structure almost all deal with a late period of development, when swimming movements are already an integral part of behaviour; when the most critical period in the relation between structure and function is already

past history. The few accounts at appropriately early stages of development deal chiefly with sensory or motor neurons which have peripheral processes. This applies to that of Neal (1914) on *Squalus* and to Harrison's classical account of the Rohon-Beard cells of *Salmo* (1901).

On the other hand, good descriptions of the early stages of behaviour are also rare. The few that exist, such as that of Wintrebert (1921) on *Scylio-rhinus*, are difficult to assess in the absence of knowledge of the contemporary nervous structure.

In fishes we lack an adequate picture of the sensori-motor arc in the nervous system during the period when locomotor movements or those of the pectoral fins and visceral arches are being developed. In cyclostomes the position is even less satisfactory. For this group there is no description of the relation between the neurons of the spinal cord in the young. Yet such a description in vertebrates so near the prototype is clearly fundamental.

The present paper attacks this problem in the Petromyzontia. The detail of the nervous structure will be described at the period in which locomotion by swimming movements has just been achieved. The animal is then at a stage roughly comparable with the stages described in *Amblystoma* by Youngstrom.

### Previous Work on the Petromyzontid Nervous System

The extensive literature on the neurology of lampreys has been well summarized in Kappers-Huber-Crosby (1935). The descriptions of the brain are relevant to a study of the spinal cord in that they show that the brain, at least, appears to be organized in a very primitive fashion. The simple form of the brain in the young ammocoete can be seen from von Kupffer (1906), figs. 47 to 57. The primitive and surprisingly unspecialized neural architecture and tracts of the adult brain are clearly described in Johnston (1902). Later work, such as that of Barnard (1936), Woodburne (1936), and Pearson (1936), has confirmed his conclusions.

Work on the neurology of the brain has been mainly devoted to adult or advanced ammocoete preparations. For instance, Tretjakoff (1909*b*) gave an extensive account of the brain of ammocoetes of length 10 to 18 centimetres. In passing, it is important to note that fig. 43, Tretjakoff (1909*b*), is reproduced in Kappers-Huber-Crosby (1935), fig. 316, where it is wrongly described as part of the brain of an 'Embryo'. But Larsell (1947) has recently described some parts of the brain in younger ammocoetes: the smallest of his silver-impregnated specimens was 42 mm. in length.

In contrast to the brain the spinal cord of the older ammocoete and adult lamprey is not of the form to be expected in a prototype, and the function and homology of the constituent neurons are difficult to understand. Kolmer (1905) and Tretjakoff (1909*a*) have been the chief contributors on the subject. Their conclusions are discussed by Kappers-Huber-Crosby, but the original figures should be consulted. The unusual characters of the spinal cord may be summarized as follows. The cord is so broad and flat as to be almost

ribbon-like; the positions of dorsal, lateral, and ventral funiculi are therefore abnormal. Dendrites of motor neurons extend across their own half of the spinal cord, and may extend to the contralateral side, either above or below the neural canal: the peripheral motor fibre is derived from a collateral of a longitudinal fibre. The chief form of intercalary cell is very large: its dendrites extend into the contralateral dorsal funiculus, while its axon runs longitudinally and then crosses into the contralateral ventral funiculus. Giant fibres descend in the ventral and lateral part of the cord from the giant cells of Müller in the midbrain and hindbrain; these are believed (e.g. by Stefanelli and Camposano, 1946) to be co-ordinating fibres comparable to the Mauthner fibres of teleosts and urodeles, but they occupy a considerable part of a transverse section of the cord. In a dorso-medial position in the grey matter are found some very large cells, termed *Hinterzellen*, whose axons run in the dorsal funiculus. There are normal dorsal ganglia, but the dorsal and ventral roots do not unite in the usual manner.

Two points in the description of the cord of adults and the older larvae require special attention. Firstly, in a Viennese journal, Sigmund Freud (1877) described somatic-sensory fibres which he claimed and figured as originating from the Hinterzellen, i.e. from cells of the dorsal horn. Now in the larvae of fishes and amphibia there are large neurons within or just above the dorsal part of the spinal cord which have the relations of somatic-sensory dorsal ganglion-cells; these neurons are termed Rohon-Beard cells. If Freud's figures were correct, there would be a strong presumption that the Hinterzellen are homologous with Rohon-Beard cells, especially since Rohon-Beard cells are persistent in many adult teleosts. This relation between the Hinterzellen and the dorsal root was subsequently denied by Tretjakoff (1909a). But it was again described by Beccari (1909), who supported his case with some very clear figures.

Secondly, there is a well-developed Commissura Infima Halleri at the anterior limit of the cord. This commissure was described in the lamprey by Johnston (1910), but his paper was not mentioned by Kappers-Huber-Crosby. The importance of the functions of the Commissure of Haller are emphasized by Herrick (1944), and it can be seen, from a comparison of Johnston's account of its structure with the description by Herrick (1908) of its relations and its functions in fishes, that in lampreys this commissure is probably concerned with a correlation between the left and the right spinal somatic-sensory components, and also between these and the cranial nerves.

Turning to the embryo and the young pride, we find three descriptions of the large dorsal cells in the spinal cord; the first two of these have been overlooked in Kappers-Huber-Crosby's account. Kupffer (1894) showed a 'Rohon'sche' cell, i.e. a Rohon-Beard cell, lying just dorsal to the cord in a 3-mm. embryo. Studnicka (1895) figured Hinterzellen in *L. planeri* of lengths between 3 mm. and 30 mm. In his younger stages, the Hinterzellen now lie just below the dorsal surface of the cord; in the older stages, they have sunk still farther into a position ventral to the dorsal sectors of the white matter.

The axon runs longitudinally in the white matter; the peripheral process appeared to him to be independent of the dorsal root, and free from metameric organization. Studnicka concluded that the Hinterzellen were probably motor in function. Beccari (1909), using a silver impregnation, described the same cells in prides of 20 mm. His account confirms that of Studnicka, except that he found that the peripheral fibre left the cord as part of the dorsal root. It seems clear from the three papers that the large dorsal cells are true Rohon-Beard neurons, for they have very large cell-bodies which begin in a very dorsal position in the cord, an axon running longitudinally in the dorsal white matter, and a peripheral fibre which is presumably sensory, since in the older stages it accompanies the other dorsal-root fibres.

The only other type of neuron described in the spinal cord of these stages of *Lampetra* is the motor neuron. Kupffer (1890) described the first motor axons running from the spinal cord to the myotomes in 3-mm. embryos (his fig. 81).

The peripheral nerves of the trunk in the newly hatched pride have been described by Sagemehl (1882), Shipley (1887), Dohrn (1888), and von Kupffer (1890). Von Kupffer states that the ventral spinal nerve, on leaving the cord, passes ventrally between the notochord and the inner face of the myotome. He describes the dorsal nerve as emerging from the cord and dividing into a lateral and a median branch: the median branch turns ventrally, to pass between notochord and myotome, while the lateral branch passes outward across the upper surface of the myotome. The dorsal ganglion-cells are figured as lying on the course of the median branch of the dorsal nerve, e.g. his figs. 81 and 82.

The relation between the dorsal ganglion and the dorsal nerve was a matter of dispute between these authors. But it would have been difficult to establish the facts with certainty by the aid of the stains they were using: von Kupffer, for instance, used borax-carmine. Certainly, the cells of the dorsal ganglia of the spinal cord are still in a very early stage of development (see Shipley). Methylene blue preparations of peripheral nerves in much older ammocoetes have been described by Tretjakoff (1929). His fig. 25 shows a dorsal spinal nerve dividing into four branches. Two of these branches correspond to the median and the lateral branches of von Kupffer's account: but the other two run, one dorsally and the other dorso-laterally, quite clear of the myotome to reach the skin. His fig. 26 shows a motor neuron.

The position reached by previous work is, then, that in the newly hatched larva the sensory and motor somatic nerves have reached their end-organs; that the sensory fibres are at least partly derived from Rohon-Beard neurons or other intramedullary neurons very like them; and that the dorsal ganglion-cells on the other hand are at an early, probably only neuroblastic, stage of development. In short, the peripheral relations of the nervous system in the trunk have already been considerably elucidated. On the other hand, very little is known about the neurology of the spinal cord. Some of the central

relations of the sensory neurons might be deduced from Studnicka's work and Beccari's work on older animals. But nothing is known about internuncial neurons and the correlating mechanism; nothing is known about the development of descending fibres from the brain which might by this stage be coordinating the motor tracts; nor have either the dendrites or the longitudinal processes of the motor neurons been described.

<div align="center">METHODS</div>

Two methods of staining neurons specifically were used, namely, vital staining with methylene blue and 'silver on the slide'.

Methylene blue does not appear to have been used previously on the nervous system of vertebrate embryos. It has many advantages.

1. Much younger material may be used than will adequately take other nerve-specific stains, or impregnations.
2. Total preparations may be made of the more transparent embryos such as *Salmo*. These show much that would be difficult to perceive or reconstruct from sectioned material. This is especially true of nervous structures which are segmentally repeated.
3. The neurons which have taken the stain may be examined either in the living animal or in permanent preparations.
4. Methylene blue and Golgi preparations have many similar characteristics, as Polyak (1941) has pointed out. The sections may be relatively thick, $30\mu$ to $60\mu$. Only a limited number of neurons are shown up, but these take the stain throughout, in nucleus, cell-body, axon, and dendrites. Methylene blue and Golgi sections are therefore relatively simple to interpret.

In the case of lamprey material, the presence of opaque white food-material in the cells of the younger embryos makes it difficult to observe the central nervous system in the living animal by means of methylene blue.

Silver was used to complete the picture given by the methylene blue. Since silver impregnates all neurons a more quantitative account is possible. Its use also ensures that no entire category of neuron shall be omitted from any reconstruction, for it is exceptional for methylene blue to colour some categories, e.g. the Müller and Mauthner giant fibres.

A silver method where reduction takes place on the slide is more easily controlled than one of the Ramón y Cajal methods, and is therefore particularly suited to very young material, for which adjustments in any technique are usually necessary.

As a control method embryos fixed in Susa were stained by a variety of standard techniques. The most effective was Mallory's phosphotungstic acid haematoxylin, which gave good differentiation and also picked out the neuroglial cells.

*Methylene blue.* The stain may be applied either as a coloured solution, or as a colourless solution of Rongalit-methylene blue. The former appears not

to penetrate so well. The results with Rongalit-methylene blue are, on the other hand, less predictable, so that it is less easy to determine beforehand what nervous elements shall take the stain. In each case, the quality of the methylene blue is important. In this work, Grübler's methylene blue for 'Vitalfärbung, nach Ehrlich' was used.

The coloured solution was applied to embryos such as trout and lamprey at about 10° C. at dilutions of about 1:1000. The embryos were completely immersed. The first staining, apart from any tissue which might have been cut or damaged, usually occurred after 2–5 hours. Usually nerve-cells, especially those with long peripheral fibres, were the first to appear blue. Coloration is intense, so that there is usually excellent contrast between stained and unstained elements. Where there is a large yolk-sac, as in the trout, it is advisable to cut away the ventral part so that the dye can reach the coelom directly.

Rongalit-methylene blue was applied by the method used in this laboratory by Dr. C. F. A. Pantin, F.R.S., and by Dr. J. E. Smith upon invertebrate material. Their suggestions concerning its use were very helpful. The method is described by Smith (1946).

Whether using methylene blue or its colourless leucobase, the resulting preparations may be made permanent. The procedure has been developed from that of Cole (1936). Only on neurons which are still intensely stained should fixation be attempted, because some time elapses before the fixative affects the dye. *Stages 1–6 are carried out at between 0° and 3° C.*

1. The embryos are fixed for 2 hours in 8 per cent. ammonium molybdate. If it is necessary to fix overnight, difficult material should be given a preliminary fixation in saturated ammonium picrate *of 5 minutes' duration.*
2. Wash in water for 1 minute.
3. Transfer to the following *n*-butyl alcohol mixtures in the Lang (1937) series for 30–40 minutes each: 30, 57, 82, 91, 97 per cent. total alcohol.
4. Leave in absolute *n*-butyl alcohol for 2–12 hours.
5. Transfer to equal parts of methyl benzoate and *n*-butyl alcohol, then to pure methyl benzoate, for 1 hour in each.
6. Transfer to a mixture of methyl benzoate and liquid paraffin for 1 hour.
7. Transfer to ice-cold liquid paraffin. The embryos may now be allowed to come to room temperature. They may be left in liquid paraffin indefinitely, before they are examined as total preparations, or embedded in wax and sectioned.

The purpose of this procedure is to avoid the leaching effect of water or ethyl alcohol so far as possible. The following additional points may be helpful. (i) Longer immersion in ammonium picrate, as in the technique of Bethe (1898), causes the nerves to appear green and the background yellow. (ii) Diethylene dioxide has been used for dehydrating methylene blue material, but has not been found so satisfactory as the *n*-butyl alcohol technique. (iii) If the specimens are not to be sectioned, they may be brought from

methyl benzoate through a mixture with xylene and Canada balsam into balsam. Balsam preparations of *Salmo* embryos with stained neurons have kept excellently for the period of the war.

*Silver.* Embryos, narcotized in weak urethane, are immersed for 2 days in the alcohol-chloral hydrate fixative used by Nonidez (1939). They are then dehydrated with 57, 82, 91, 97 per cent., and absolute alcohols. These alcohols are made up according to the procedure of Lang (1937), which utilizes the properties of *n*-butyl alcohol, and is less damaging to delicate tissue than ethyl alcohol dehydration. The specimens are brought slowly through methyl benzoate, then liquid paraffin, and then imbedded in paraffin wax.

The methods of Nonidez and Lang enable considerably better impregnation to be achieved than is attained on similar material by more orthodox procedure.

The subsequent treatment followed the technique of Holmes (1943), which is one of the best of the many variations of the method of Bodian (1936). On the ammocoete material, good results were obtained using a 1 : 30,000 solution of silver nitrate in distilled water, buffered at pH 8·3 at 37° C. for 2–3 days.

Bayer's German pre-war Protargol was also used in place of silver nitrate, and gave good results. This form of Protargol is now difficult to obtain. The Protargol should either be used with addition of metallic copper, as in Bodian's method, or should be buffered to about pH 8·0 with the borax-boric acid buffer used in the Holmes method.

Professor J. E. Harris, Department of Zoology, Bristol University, has given me much important advice upon the silver procedure.

Counterstaining, e.g. with Orange G, is sometimes an advantage.

## MATERIAL

The prides used in this study were between 7 and 10 mm. in length. They correspond to the developmental stage VIII of Hatta (1896), in which the stomodaeum has just opened. The general morphology of the central nervous system of these specimens closely resembles that figured by von Kupffer (1906) for the 6-mm. animal: no appreciable change toward the form described by him for the 20-mm. ammocoete has been observed.

The material was obtained from two sources:

(i) Prides of *Lampetra planeri* from a tributary of the Lymington river, in the New Forest. I am greatly indebted to Mr. A. R. Hockley, Lecturer in Zoology, University of Southampton, for the living and the fixed material from this source, and also for demonstrating to me his methods of collection.

Two batches from the New Forest were used for the silver preparations. Batch 'A', average length 7 mm., was fixed 10 days after fertilization. Batch 'B', average length 8 mm., was fixed 16 days after fertilization.

(ii) *Lampetra* prides, of 8–10 mm. in length, received alive from Professor J. E. Harris. These were used for methylene blue preparations.

Sagittal and transverse serial sections were prepared of both batches for the silver method: horizontal sections were prepared only of the 'B' batch.

Impregnation and differentiation of nervous tissue both in brain and spinal cord and peripherally was satisfactory in all sections.

## The Nervous System of the Trunk Region of Young Ammocoetes

*Sensory System*

The chief sensory system at this stage consists of neurons having the form and relations of Rohon-Beard cells: they will be described under that name.
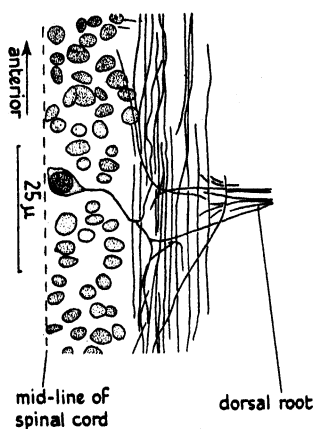


| | |
|---|---|
| Fig. 1 | Fig. 2 |

Text-fig. 1. Horizontal section, 16μ, silver impregnation; from anterior trunk region of 16-day *Lampetra*.
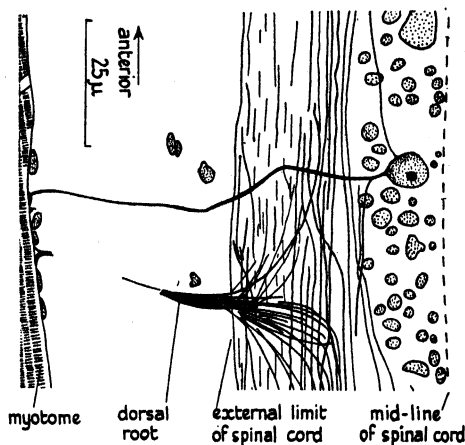
Text-fig. 2. As Text-fig. 1, but nearer the head. These and the succeeding figures were drawn by means of a camera lucida, using ×45 and ×95 objectives.

*Rohon-Beard neurons.* The cell-body lies in the dorsal grey matter, with its median surface close to the neural canal. It is rounded and large, its diameter being about 6μ, or about twice that of any other type of neuron in the dorsal part of the spinal cord. Each neuron has three large processes: two turn outward into the white matter, where one fibre proceeds towards the head, and the other caudally: the third process runs outward, leaves the cord at the dorso-lateral edge, and runs to the somatic muscle or to the skin (Text-fig. 1).

At anterior levels the peripheral fibre usually lies in about the same transverse plane as the cell-body, and the cell-body is unipolar, as in Text-fig. 1. At posterior trunk levels the peripheral fibre leaves the cord some distance caudal to the cell-body, and is derived, as a collateral, from the descending fibre. The descending and ascending fibres originate from the cell-body separately, so that at posterior trunk levels the cell-bodies are seen in horizontal section to be consistently bipolar.

The longitudinal fibres can be traced for a distance equal to several somites: they give off slender branches, which appear to be quite short. The dorsal

white matter seems at this stage to be largely made up of the longitudinal Rohon-Beard fibres. The ascending fibres of Rohon-Beard cells lying near the head appear to run into the Descending Trigeminal tract of the medulla. The peripheral fibre normally runs out in the dorsal root, of which it is the largest, and at this stage the most numerous, component. Such a typical condition is seen in Text-fig. 1. But frequently one fibre runs out separately from those in the dorsal root. When such a separation is in the horizontal plane the outlying fibre has a quite normal course after reaching the endo-chondral layer (Text-fig. 2).

The cell-bodies lie in two rows, one on each side of the midline, for the whole length of the trunk. They are at various dorso-ventral levels: some project above the general outline of the grey matter, while others are as far ventral in it as the area which would in gnathostomes contain the viscero-sensory component. No difference in the peripheral destination of the more dorsal and the more ventral cells could be observed. Presumably, in older ammocoetes, the cells of the two rows would settle into a single dorso-ventral level.

The dorsal root passes through the endochondral tissue at the interseg-mental position, opposite the myocomma. On the outer side of the endo-chondral layer the root traverses the area of the dorsal ganglion, which lies at a dorso-ventral level midway between dorsal and ventral roots. Some Rohon-Beard fibres of the dorsal root pass beside or even between the cells of the dorsal ganglion without being in any way connected with them. This independence could be seen in transverse and parasagittal silver sections; it is probably true of all Rohon-Beard fibres, although the dorsal ganglia are so compact that it was not possible to be certain of the independence of every one of the many Rohon-Beard fibres examined (Text-fig. 5a). In methylene blue preparations no stained dorsal ganglion-cells have been found: stained Rohon-Beard fibres are seen to pass from the endochondral layer directly into the interface between successive myotomes.

Between the myotomes the Rohon-Beard fibres spread out and branch in lateral or ventral directions. The larger branches reach the skin. Small branches ending as proprioceptors on the ends of the myofibrils remain in the myosepta. No instance was found of branches penetrating in a longitudinal direction to end in parallel with the myofibrils (Text-fig. 3).

The Rohon-Beard fibres to the ventral part of the myotome and to the skin of the ventral surface do not penetrate between myotomes until they have passed well below the notochord: for this reason a parasagittal section just lateral to the notochord shows both the motor nerve and the ventrally directed Rohon-Beard fibres. The motor nerves lie in a mid-segmental and the Rohon-Beard fibres in an intersegmental position in remarkably consis-tent fashion through the whole length of the trunk.

On reaching the skin the Rohon-Beard fibres divide further; some sub-divisions reach the dermis and may penetrate the basement membrane of the

epidermis (Text-fig. 4). Others continue down the outer side of the inter-somite, where, by meeting other similar branches, they become an appreciable aggregate of fibres.

Just as some Rohon-Beard fibres run out separately from the dorsal root, slightly caudal or cephalad to it, so others proceed outward independently and dorsally to the dorsal root. Most of these fibres reach the dorsal tip of the myotome, where they cross the muscle in an intersegmental position, or pass round the top of the myotome to reach the skin. Since such fibres run



FIG. 3                    FIG. 4
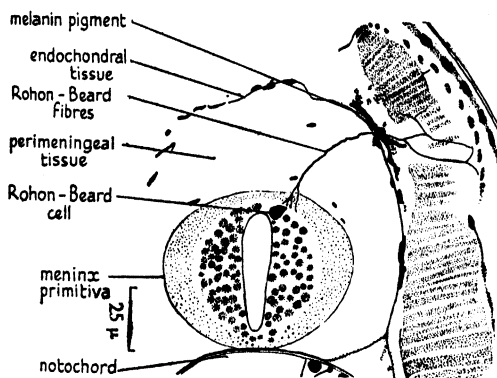
TEXT-FIG. 3. Transverse section, 16 μ, from a 10-day *Lampetra*; silver method. Shows the peripheral course of some Rohon-Beard fibres.
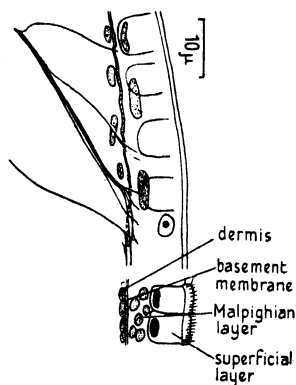
TEXT-FIG. 4. Transverse section, 16 μ, from a 10-day *Lampetra*; silver method. Exteroceptive terminations of Rohon-Beard fibres. (Below, sketch showing appearance of the skin with standard histological methods.)

singly, and since they are clear of the dorsal ganglion on the whole of their course, they are easy to examine (Text-fig. 5b). Large branches of these fibres reach the skin, but the smaller branches turn back to end on a myofibril. Such Rohon-Beard fibres correspond closely to those described by Coghill in *Amblystoma*.

Others of the separate, latero-dorsally directed Rohon-Beard fibres do not reach the myotome at all, but pass from the dorsal surface of the spinal cord through the endochondral layer at only a slight angle to the vertical, so that they reach the skin of the dorsal surface. This third type of Rohon-Beard cell has been noted previously by Studnicka, Tafel III, fig. 11. He regarded the type as aberrant: but it occurs in small numbers at all trunk levels, and is evidently the only sensory neuron which innervates the dorsal areas of the skin in the young pride (Text-fig. 5c).

The Rohon-Beard fibres which proceed independently of the dorsal root are confined, like those in the dorsal root, to an intersegmental position.

When they occur at an intersegment in pairs they are arranged symmetrically about the midline. They are probably the 'pathfinders', the earliest sensory neurons to have developed at that intersegment, and neither of the independent types should be regarded as aberrant.

*Dorsal Ganglion-cells.* Some of the cells of the dorsal ganglia are round or pear-shaped neuroblasts: others are bipolar neurons which have slender peripheral and central processes. Neither form of process could be traced to its
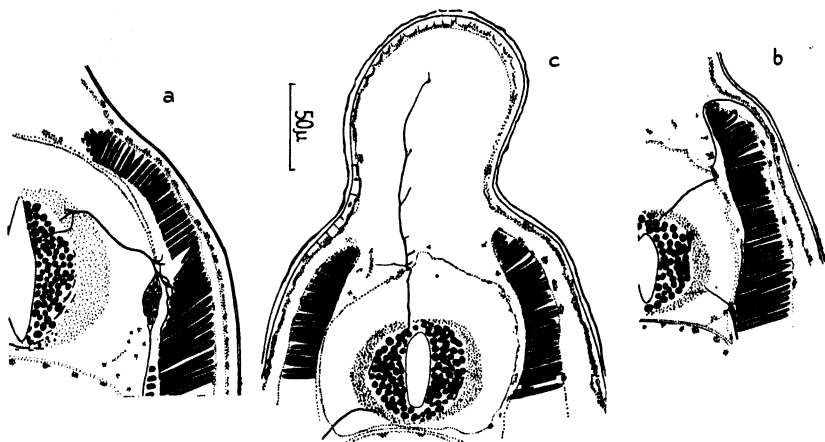


Text-fig. 5. *a.* 12 μ, silver method, 16-day *Lampetra*; Rohon-Beard fibres in the dorsal root. *b.* 16 μ, silver method, 10-day *Lampetra*; Coghill's type of Rohon-Beard fibre. *c.* 16 μ, silver method, 10-day *Lampetra*; dorsal type of Rohon-Beard fibre.

destination, but there are several fine fibres in each dorsal root, which have presumably come from the dorsal ganglion-cells. The peripheral process is usually directed ventrally, but was never traced far enough to show to which functional component these early dorsal ganglion-cells belong.

### Correlating System

*Large Internuncial Cells.* In the lateral part of the grey matter there occur some large cells whose axons run to the ventral motor tract, and whose dendrites are in clear relation with the dorsal tracts of the same and also of the opposite side of the cord (Text-figs. 6 and 7).

Dendrites to the lateral, dorso-lateral, and dorsal areas of the white matter arise from the cell-body separately. Those to the lateral area are short: those to the dorso-lateral area are longer. Dendrites to the dorsal areas have a characteristic course: they run directly dorsally, entering the white matter at an oblique angle: they then turn medially in a wide curve which keeps them clear of the Rohon-Beard cell-bodies. Most of the dendrites to the dorsal area continue medially, so that they enter the contralateral dorsal area by way of the dorsal commissure. The contralateral process runs directly across the

cord, so that the cell-body and the crossing dendrite may appear in a single section, as in Text-fig. 6. The contralateral process can also be seen in sagittal sections at 20μ, for it is quite large, and can be followed from the cell-body to the midline by adjusting the optical section. The axon enters the ventral area of the white matter, where small collaterals branch off into the ventrolateral area. The main process turns forward to run with the longitudinal fibres. Although it is now near the midline, it does not cross to
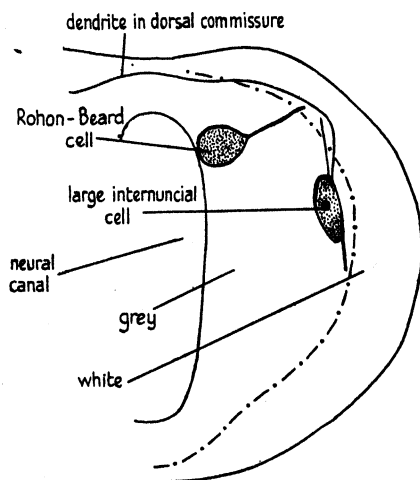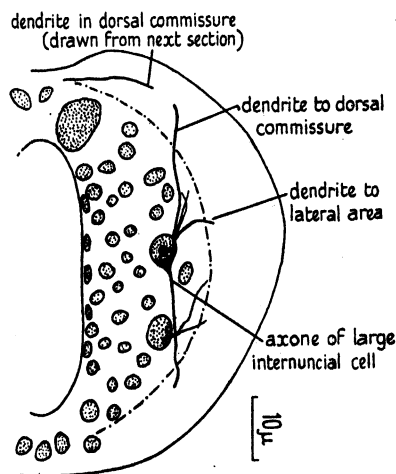


FIG. 6                                    FIG. 7

TEXT-FIGS. 6 and 7. 16μ, silver method, 10-day *Lampetra*; dendrites and axons of the large internuncial cell.

the contralateral motor areas, and no collaterals from the axon have been observed to cross (Text-fig. 8).

These large internuncial cells usually occur singly, one per segment, somewhat caudally to the origin of a ventral root, with which the axon is no doubt in synaptic relation. But the distribution of these cells is not quite so constant as might be inferred from the region figured.

*Small Internuncial Cells.* The Rohon-Beard and the large internuncial cells are only a small proportion of the nerve-cells in the dorsal half of the cord. Many of the remainder are neuroblasts, some of which do not appear to have yet formed any process from the cell-body: those which do have dendritic and axonic processes have not, however, achieved a definitive or constant structure, though they all appear to be developing into small internuncial cells which link the ipsilateral sensory and motor fields and which have no longitudinal process. Cell-bodies of this type of neuron lie at the dorso-lateral edge of the grey matter. There is a thick, 'protoplasmic' rather than fibrillar axon, which runs down between the grey and the white and ends on

the cell-body or immediately adjacent dendrites of a motor cell. The dendrites are short, and are usually directed into the dorso-lateral white, but they are diffuse in form and very lightly stained. It is probable that the dendrites are growing towards the incoming central fibres of dorsal ganglion-cells, which we know to be at the same stage of outgrowth. The contrast between the diffuse, almost amoeboid form of these cells, and the constant and well-defined form of the large internuncial neurons is marked.

*Oblique Fibres.* At fairly regular. intervals along the mid-trunk region of the cord a single large fibre swings out of the dorsal funiculus and descends
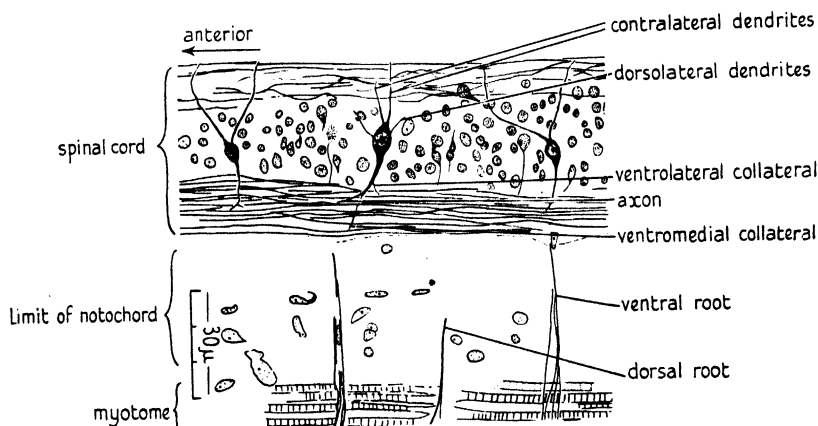


TEXT-FIG. 8. Parasagittal section, 20μ, from a 10-day *Lampetra*; protargol silver method. Three successive large internuncial cells, showing their position relative to the motor roots.

obliquely towards the ventral funiculus: it reaches the latter about four somites more caudal than its origin from the dorsal funiculus: the fibre continues caudally in the inner and medial part of the ventral white, internal to the Müller fibres. The oblique fibres can be picked out in transverse sections, where it can be seen that during the oblique part of their course they pass medial to all the longitudinal fibres of the lateral white. In parasagittal sections the oblique fibres stand out sharply, because the other large fibres all run longitudinally in the cord. Since they are on the inner side of the white matter they necessarily pass close to at least one of the large internuncial neurons: but no synaptic relation was observed between them. Individual oblique fibres were followed towards the head in the dorsal white matter; but the cell of origin has not yet been found, nor has any large cell of different form from the Rohon-Beard cell been noticed in the dorsal white at an interval of one per four somites (Pl. Ic).

In horizontal sections of the ventral part of the cord, the oblique fibres are seen to continue in a ventral and caudal direction until they are directly medial to the Müller fibres; they may run parallel with the Müller fibres for a short distance, but they soon swing abruptly out of line, turning ventrally

and medially to reach the ventral white of the opposite side by way of the ventral commissure. Each oblique fibre turns to run parallel with the contra-lateral Müller fibres, but becomes rapidly attenuated and terminates (Text-fig. 9). The oblique fibres seen in horizontal section appear at fairly regular intervals, about four somites' distance separating successive fibres of the same side. Those of the two sides are not paired. Oblique fibres have not been seen in either sagittal or horizontal sections to occur in the anterior third
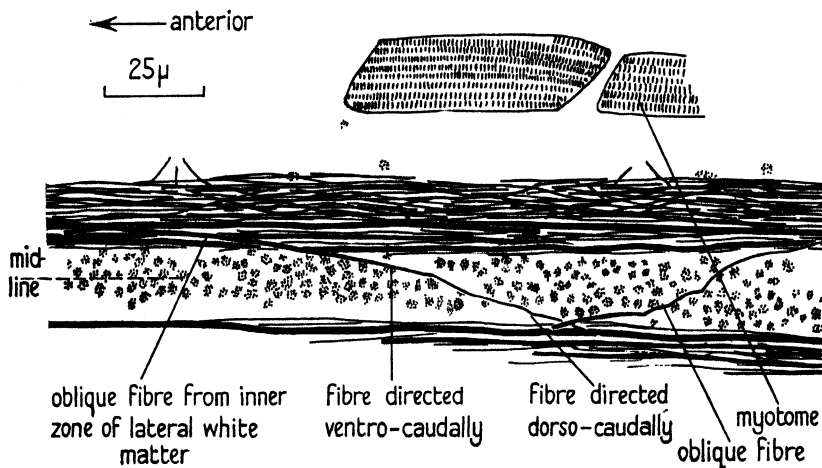


TEXT-FIG. 9. Horizontal section, $16\mu$, 16-day *Lampetra*; silver method. (Plane of section is slightly more ventral towards the lower left side of drawing.) Fibres drawn as if viewed from dorsal aspect; shows the course of two oblique fibres in the ventral commissure.

of the spinal cord: it seems probable that, at this stage of development at least, they do not occur in that region.

*Cells of the Commissura Infima Halleri.* In a dorso-medial position, in the most anterior part of the cord, there are on each side of the midline about twenty neurons with characteristic bipolar, spindle-shaped cell-bodies. All are oriented transversely: from the medial end of the spindle a single dendritic process crosses the midline close to the dorsal surface of the cord to end in the contralateral dorsal part of the cord. The outer end of the spindle is formed by the axon, which runs laterally into the dorso-lateral part of the white matter (Pl. IA).

These neurons can be seen in horizontal and transverse sections of 16-day prides, when they have the form described: but in 10-day prides the cells are still unipolar neuroblasts. They occur in a length of about $60\mu$ of the cord, posterior to the choroid plexus of the medulla oblongata: the most caudal is at the same transverse level as the most anterior Rohon-Beard neurons. The diameter of the cell-body, measured across the spindle, is about one-third that of the Rohon-Beard neurons. The centre or nucleus, constituted by

these cells, lies dorsal to the ordinary cells of the dorsal grey, and median to the two dorsal funiculi, which at this level are separated from each other by this centre.

Unfortunately, the longitudinal relations of the centre are not yet known. A few of the axons can be seen to turn caudally after reaching the dorsal funiculus, while a small collateral runs anteriorly. But the details already established show that these neurons constitute by the sixteenth day a Commissura Infima Halleri and its Nucleus.

_Müller Fibres._ The axons of the Müller cells of the brain run toward the tail for the whole length of the spinal cord. In transverse sections of the
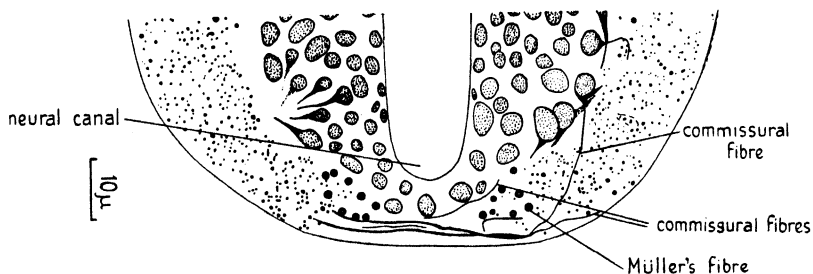


TEXT-FIG. 10. 12 μ, 16-day _Lampetra_; silver method. The ventral commissure.

trunk they are seen below and lateral to the floor-plate cells and median to the other longitudinal fibres of the ventral funiculus (Text-fig. 10).

On each side of the cord there are about eight Müller fibres which are larger in diameter than the other longitudinal fibres of the cord, and about another eight are of the same diameter as the biggest of the fibres in other tracts. Each fibre remains on the same side of the cord as its cell of origin, and closely parallel to its fellows, so that the pattern made by the Müller fibres remains constant through many transverse sections. The fibres do not divide, nor have they been seen to give rise to any collaterals.

This description agrees with the accepted account of older animals, except that the Müllerian fibres in the young pride do not have the extraordinarily enlarged diameter found in older prides, and the tract still retains the position to be expected in a motor co-ordinating system. The cells of origin in the brain show extremely well in the present material: their form and the relations of their dendrites correspond with the figures given by Tretjakoff. The Müller cells lie in two groups, one in the midbrain and the other in the hindbrain: the dendrites of the two groups bring the cells into synaptic relation with all cranial sensory nerves except the optic and olfactory. A single large cell on each side can be seen in the present material to reach most parts of the forebrain with its dendrites, and to have an axon descending to the region of the Müller cells of the midbrain. There are optic fibres in the optic chiasma at this stage; and Studnicka and Walls (1944) have stated that there is a primitive

functional lens at this period. Presumably, therefore, the Müller fibres of
the spinal cord may be affected by stimulation of any of the cranial nerves,
including those of the forebrain group.

At the most anterior levels of the spinal cord a pair of the Müller fibres,
apparently of similar cranial origin to the others, turns laterally and dorsally,
reaches a position median to the lateral funiculus, and continues caudally, one
on each side, in this position for the whole length of the trunk. This dorsal-
ward movement of one pair of Müller fibres is very striking in the young
ammocoete.



TEXT-FIG. 11. 12 μ, 16-day *Lampetra*; silver method. A primary motor neuron.

*Motor System*

*Somatic Motor Neurons.* Some of the larger axons in the ventral root can
be followed, in a single transverse section, peripherally to motor end-plates
on the myotome and centrally to cell-bodies lying in the ventral area of the
grey matter. In such cases the axon grows from the cell-body into the
ventral motor tract; from the tract its course to the motor root is horizontal
(Text-fig. 11). The majority of motor neurons are not so easily traced,
because the cell-body and the ventral root to which it contributes are in
widely separate transverse planes; but almost all the large axons can be traced
from myotome to ventral root and thence horizontally as far as the ventral
motor tract.

The motor neurons of the ventral grey area are of two forms. Those lying
in the outer part have a 'protoplasmic' area of the cell-body on the lateral
aspect of the nucleus: this area extends dorsally between the grey and the
white, giving origin, on its surface against the white, to many branching
dendrites which terminate on the lateral and ventrolateral surface of the
spinal cord. The axon originates separately, from the outer and ventral
part of the cell-body (Text-fig. 12). On entering the ventral motor tract

it turns longitudinally, giving off the peripheral fibre to the motor root as a collateral.

Neurons of the inner part of the ventral grey matter effect the same relations in the cord, but the processes from the cell-body are thin, cylindrical fibres until they reach the boundary between grey and white, when they also form a protoplasmic extension at the base of the dendrites, though it is less extensive (Text-fig. 13).

These ventral motor neurons usually have a separate process on the ventral or ventromedial surface of the cell-body (Text-figs. 10–13). This process can sometimes be traced into the ventral motor tract of the opposite side; sometimes it appears to originate as a collateral of the axon. These commissural
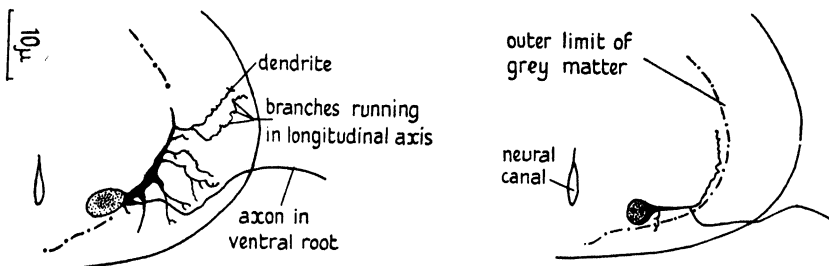


TEXT-FIGS. 12 and 13. 34μ, methylene blue method. Primary motor neurons.

fibres are best seen in cases where their whole course is through the white matter, as in Text-fig. 10. They appear to have no relation with the Müller fibres of their own side; their relation with the Müller fibres of the opposite side may be closer, but has not yet been determined. The commissural fibres usually pass ventral to both sets of Müller fibres, but sometimes they pass dorsal to those of their own side; two of this latter type can be seen in Text-fig. 16.

The neurons of the ventrolateral grey matter which are developed by this stage probably also belong to the somatic motor component, of which they would then constitute a second type. As in the case of those just described, the dendrites from neurons lying deep in the grey are thin fibres, while the dendrites of cells on the outer limit of the grey matter take origin from a broad 'protoplasmic' area of the cell-body. The dendrites reach an extensive area of the lateral funiculus, but have not yet been found to extend into the ventral funiculus. A great proportion of the branches of the dendrites run a short distance longitudinally in the lateral funiculus, so that they must represent a large part of it. Unlike the first type these neurons have some dendrites which extend dorso-laterally to come into contact with the longitudinal fibres of the Rohon-Beard cells. The axon is directed ventrally before leaving the grey matter, so that they enter the ventral funiculus close to the axons of somatic motor neurons of the first type. Instances in the second type where the cell-body, the intramedullary and the peripheral part of the axon lay in

2421.8                                                C C

the same transverse section have not so far been found. The motor neurons of the second type appear to be somewhat younger than the first type, and did not show up so well in the silver material, which was prepared from slightly young embryos (Text-figs. 14 and 15).

*Visceral Motor Neurons.* The innervation of the splanchnic muscle of the gill-region appears to be derived entirely from the vagus nerve, which can be seen on the ventral and outer side of the anterior cardinal veins. The segmental ventral branch of each spinal motor nerve of this region passes
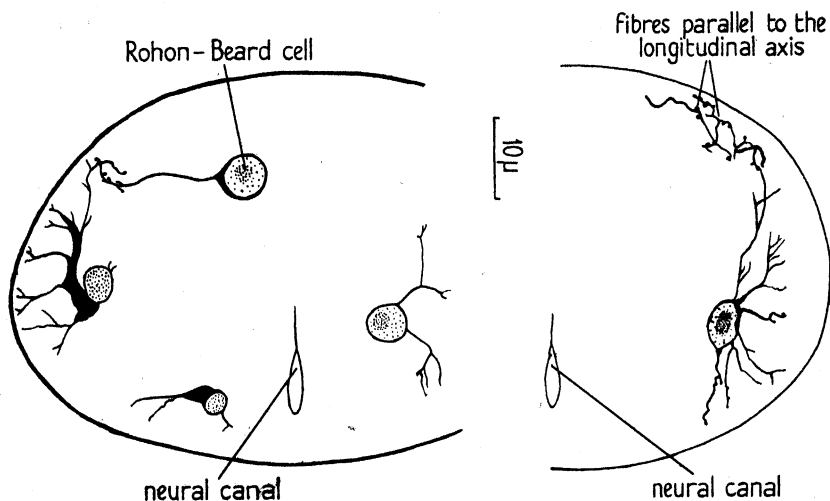


TEXT-FIGS. 14 and 15.  34 $\mu$, methylene blue method.  A second type of motor neuron, in which some dendrites reach the longitudinal fibres of Rohon-Beard neurons.

close to the vagus: but the fibres of the branch remain adherent to the myotome and none appear to join the vagus nerve or to innervate splanchnic structures directly, or to reach a sympathetic ganglion: nor have sympathetic ganglia yet been identified. It is possible that a small viscero-motor component may, however, exist and have been overlooked, because the organs lying in the area between the pharynx or the intestine and the myotomes are surrounded by stellate melanophores. Consequently, in silver preparations, the black nerve-fibres are extremely difficult to follow among the melanophores. Evidently the viscero-motor component of the spinal nerves, so far as it exists at this stage, can be concerned only with the innervation of blood-vessels, and perhaps of the anal region of the gut (Dohrn, 1888). It appears improbable that the motor neurons of the ventrolateral grey belong to the viscero-motor component.

In older *Lampetra* the hypoglossal nerve is built up by the union of ventral branches from each motor root of the pharyngeal region. These branches individually enter and run a short distance with the vagus nerve before

segregating into a hypoglossal nerve (Neal, 1897; Johnston, 1905). The association between the hypoglossal and vagal nerves is certainly brought about by the backward extension of the gill-region during development. It is interesting that at the present stage of development the association has hardly commenced.

*Ventral Commissure* (Text-fig. 10). Some of the fibres in the ventral commissure have been described already as originating directly from motor cells of the ventral grey matter. But the great majority of the fibres crossing in the ventral commissure do not arise from a cell-body at the same transverse level, but from a longitudinal fibre of the ventral funiculus. In these cases, a longitudinal fibre swings towards the midline, passing under the Müller
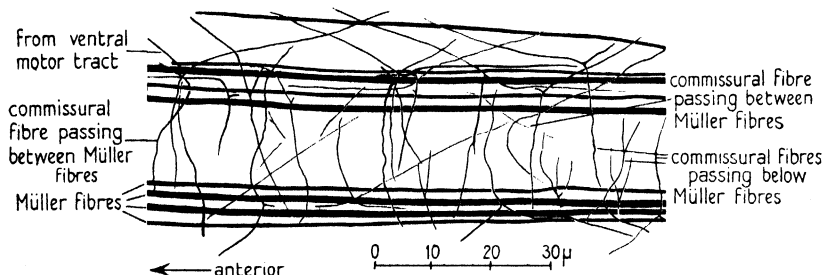


TEXT-FIG. 16. Horizontal section, 16 μ, silver method. From a region anterior to that in Text-fig. 9. The origin of the commissural fibres from the ventral tracts can be seen. (Fibres drawn as if viewed from below.)

fibres of its own side, and crosses the midline in the extreme ventral position: the fibre then becomes much thinner: it can be followed as it passes ventrally to the Müller fibres of the opposite side. Some of the fibres are too fine to be followed any farther, but others can be traced laterally and dorsally within the ventral funiculus before they also attenuate entirely (Text-fig. 16).

The crossing fibre may approach the commissure from in front or from a caudal direction. As it passes under the ipsilateral Müller fibres, it usually gives off a collateral which turns away from the midline and continues in the ventral funiculus. At the midline the crossing fibre is almost or quite in the transverse plane. It does not usually branch in the opposite funiculus. The number of crossing fibres in the ventral commissure is of the order of thirty in a length of cord equal to one somite. All, or almost all, of the fibres crossing in the ventral commissure probably originate from the somatic motor neurons, either from a process direct from the cell-body or as the termination of a longitudinal fibre in the ventral funiculus.

No outgrowth has been found from any of the floor-plate cells in the present material, at any level of the spinal cord.

In the most anterior segments of the trunk of the 16-day pride, a few of the crossing fibres in the ventral commissure appear to have a synaptic relation with the contralateral Müller fibres, to which they pass very close. These

branches on to Müller fibres are extremely minute; this detail requires confirmation on older material.

### The Tail Region

The very short tail region is omitted from the present account because the anatomical relations have proved very difficult. In particular, it has not been possible to find out the relations of the Müller fibres in the tail region.

## COMPARISON OF THE SPINAL CORD OF ADULT *LAMPETRA* WITH THAT OF THE NEWLY HATCHED LARVA

Possible homologies between the neurons described here and those in the spinal cord of older animals will be discussed only briefly, because the forms of neuron to be found in the latter were the subject of furious disagreement between Kolmer, Tretjakoff, and Johnston (1910). Knowledge of the sensori-motor arc in the adult is therefore very uncertain.

It is clear from the foregoing account that the Hinterzellen of the adult must be persistent Rohon-Beard cells. Both the Rohon-Beard cell of the young pride and the Hinterzell are very large dorsally placed intramedullary cells: they occur in two rows for the whole length of the spinal cord: no other large neuron occurs near them. They both have a large fibre ascending and a large fibre descending in the dorsal funiculus. The Rohon-Beard cell has a somatic-sensory peripheral process. A similar process is found on the Hinterzell of older prides (Freud and Beccari), but is reduced or even lost in the adults (Johnston, 1902). Correspondingly, the dorsal ganglion-cells have become the predominant source of somatic sensory peripheral fibres in the adult.

The Rohon-Beard neuron has the form of a large dorsal ganglion-cell, in spite of its intramedullary position; but in the young animal it appears to combine the functions of a ganglion-cell with those of a dorsal horn cell in the somatic sensory column. As a Hinterzell in the adult it has partly lost the first of these functions.

The large internuncial neurons of the young pride are difficult to identify among the cells described in the adult or in the older larvae; but the 'Type II motor cells' of Tretjakoff's figures appear to me to be the probable homologue. The small internuncial cells probably correspond to Tretjakoff's 'amacrine' cells.

It is difficult to say which type of cell in the adult corresponds to the oblique fibres.

The somatic motor neurons have similar relations to those described in the adult by Tretjakoff (1927). But the dendrites of these neurons do not have the bizarre arborizations at this stage, which they have acquired in the adult.

The relatively immense Müller fibres of the adult correspond in the young pride to more normal-sized fibres confined to the area in which the medial longitudinal bundle is found in Gnathostomes.

Although the individual neurons of the Commissura Infima Halleri are less developed than Johnston (1910) found them in the adult, the centre and commissure as a whole has established by this stage its relation with the brain and with the spinal cord.

In short, the spinal cord of the newly hatched ammocoete does not exhibit the unusual characters of morphology and of the shape and relations of the constituent neurons which make it difficult to regard the cord of the adult as a prototype of the spinal cord of gnathostome vertebrates.

### COMPARISON WITH SPINAL CORD OF GNATHOSTOME EMBRYOS

Reference is particularly made to Coghill's and to Youngstrom's accounts of *Amblystoma*. But the result would not be different if the comparison were made with earlier work on fish larvae, such as van Gehuchten's account of *Salmo* alevins (1895). Similarities between the *Amblystoma* larva and the young pride are:

1. There is a well-defined central grey and an outer white matter. The spinal cord is more or less cylindrical. The dorsal root, dorsal ganglion, and ventral root occur in corresponding positions.

2. The Rohon-Beard neurons are organized in the same relation to other neurons, occupy the same position, and have the same form. (Cf. Coghill, 1929, figs. 9, 10, and 11.)

3. The 'dorsal intercalated' neuron is probably homologous with the large internuncial cell of the young pride. The only notable difference is that the latter has not yet developed any process crossing in the ventral commissure. (Cf. Youngstrom, 1940, fig. 1, and Text-fig. 17 of this paper.)

4. Two forms of somatic motor neuron are found in both larvae. The primary motor neuron of *Amblystoma* is similar in all essentials to the motor neuron in the ventral grey of *Lampetra*: both have a 'protoplasmic' extension between the grey matter and the white and have dendrites extending through the lateral and ventrolateral white areas; in both the peripheral fibre is given off as a collateral of a fibre in the ventral motor tract; both have a process into the contralateral ventral funiculus; in both the cell-body does not usually lie at the same transverse level as the ventral root. Possibly they both have the same functional relation to a giant-fibre system descending from the brain. For the primary motor neuron of *Amblystoma* and of other anamniote Gnathostomes using the trunk-muscle as the locomotor organ, including teleosts, dipnoans, and anuran tadpoles, is in synaptic relation with Mauthner's fibre which originates in the contralateral hindbrain. In *Lampetra* the Müller fibres are derived from cells in the ipsilateral part of the brain, but the fibres appear to be developing a synaptic relation with the ventral motor neurons of the *opposite* side. If this should be established the Müller fibres might prove to have the same function in the trunk region of the lamprey as is effected by Mauthner fibres in fishes.

A more dorsal group of somatic motor neurons has been described by Youngstrom, the 'secondary' type. They also probably occur in the

other Gnathostomes of the anamniote division. They are concerned with local movements, while the primary type effect the total movements. Movements of paired limbs, independent of trunk movements, are believed to begin when the secondary type first appear at the appropriate level of the cord.

The ventrolateral group of motor neurons in *Lampetra* are probably also somatic motor, and have some characters in common with the secondary type in *Amblystoma*. They are in synaptic relation with the afferent neuron, they have apparently no relation with the longitudinal giant-fibre system, nor with the ventral tracts of the opposite side of the cord, and the axon is very slender. But the ventrolateral group have not been investigated in sufficient detail for these similarities to have much weight. Their relations with other neurons at present suggest that they are concerned with some very local form of movement.

The Commissura Infima Halleri has not apparently been described in very young urodeles: it is not mentioned in Coghill's summary (1929) of his work. In amniotes the commissure is considered to correlate the viscero-sensory columns of the two sides: it was regarded as having that function in the adult *Lampetra* by Johnston (1902). But Herrick (1908) showed that in fishes the commissure has a viscero-sensory and a somatic-sensory component: in fish such as the trout the somatic-sensory part of the commissure is the more important. In the trout embryo the commissure is developed at a very early stage, before the embryo can swim, and when the viscero-sensory system of the cord has hardly begun development. It is legitimate to suppose that in the young *Lampetra* the Commissura Infima is an important part of the correlating mechanism involved in swimming movements.

### THE SOMATIC SENSORI-MOTOR ARC IN YOUNG PRIDE (Text-fig. 17)

It seems clear that at this stage the Rohon-Beard cells are the chief sensory mechanism. They receive stimulation from sensory endings of very simple nature in the skin and, to a less extent, in the muscle. There are no muscle-spindles nor any proprioceptors in parallel with the myofibrils. Some Rohon-Beard neurons are entirely exteroceptive in function. Centrally, the impulses of sensory origin in the trunk pass up and down the trunk without any strict segmental organization.

The excitatory impulses can pass from the Rohon-Beard tract to the Commissure of Haller or to the Descending Trigeminal centre, thereby affecting either the other side of the trunk or the brain: or the impulse may pass to motor neurons by way of the large internuncial cells, or directly through the dendrites of the ventrolateral motor neurons, or by way of the oblique fibres. Of these paths the most developed at this stage, and that about which most detail has been elicited, is that through the large internuncial cell. But even here it is difficult to see how an apparently simple locomotor system should be related to so complex and precisely organized a system of dendrites, axons, and axon collaterals.

Consideration of the dorsal ganglion-cells, small internuncial cells, and ventrolateral motor cells is not profitable until slightly older material is available, in which these types of neuron would be more differentiated. Equally, the earliest stages in the development of the somatic sensori-motor arc are not covered in the present study.
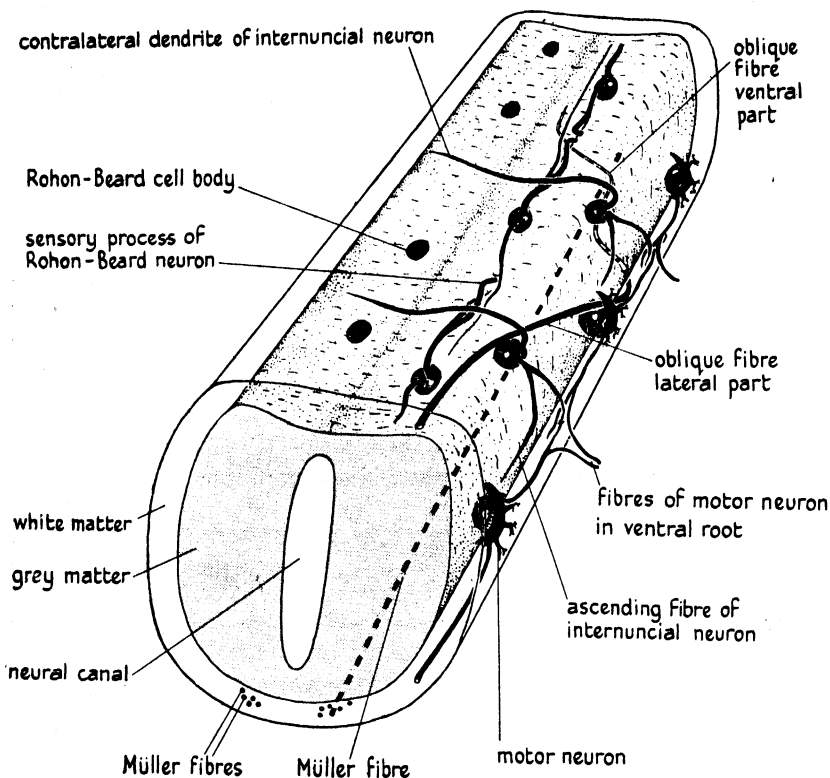


TEXT-FIG. 17. Stereogram of spinal cord of *Lampetra*, 10–16 days after fertilization, to show the relations between some of the neurons described. The figure is constructed to show a length of cord viewed from an anterior and dorsal position.

Clearly there is already in this stage of the ammocoete a complex and precisely organized structural pattern, both of the form of the neurons and of their relation to each other. Any satisfactory description of the function of this nervous system must account for the intricacies of this pattern.

I wish to thank Professor James Gray, F.R.S., for his kind encouragement and advice on problems of neuro-embryology, and Professor J. E. Harris, Department of Zoology, Bristol University, for his helpfulness in considering the problems of function which are implicit in the description of nervous

anatomy, and also for some essential advice on problems of neurological technique. I also wish to thank Dr. J. E. Smith for the construction of Text-fig. 17. Finally, I am greatly indebted to Mr. A. R. Hockley, University College, Southampton, for the collection and preservation of most of the specimens on which this account was based.

### SUMMARY

1. A method for the study of the nervous system of vertebrate embryos by methylene blue vital staining is described. A reliable technique for rendering the preparations permanent is described.

2. An adaptation of the silver 'on-the-slide' method is given.

3. Three types of sensory intramedullary neuron are described in the spinal cord of recently hatched ammocoetes, or prides, of *Lampetra planeri*. All three are regarded as types of Rohon-Beard cell.

4. Four contemporary correlating types of cell are described in the cord: large internuncial neurons with a dendritic system which reaches the contra-lateral dorsal funiculus; cells of the Commissura Infima; oblique fibres, descending caudally from the sensory to the motor tracts; and small inter-nuncial neurons with short dendrites.

5. The relations of the Müller fibres in the trunk are described in part.

6. Two types of motor neuron have been found; the more fully developed corresponds to the primary motor neuron of aquatic larvae of other anamniote vertebrates.

7. The peripheral fibres of the somatic system of the trunk are described.

8. The neurological pattern revealed is compared with that in adult *Lampetra*: the divergences from the vertebrate pattern found in the cord of the adult are not found in the young ammocoete, which in this, as in so many respects, is a good prototype of gnathostome vertebrates.

9. The probable functional pattern is compared with that found in a similar stage of *Amblystoma*.

Neurons of the correlating and motor system appear not to have been described before in ammocoetes less than 1 year old.

### LIST OF REFERENCES

BARNARD, J. W., 1936. J. comp. Neurol., **65**, 503.
BARRON, D. H., 1944. Biol. Rev., **16**, 1.
BECCARI, N., 1909. Monit. zool. ital., **20**, 308.
BETHE, A., 1898. Anat. Anz., **12**, 348.
BODIAN, D., 1936. Anat. Rec., **65**, 89.
COGHILL, G. E., 1914. J. comp. Neurol., **24**, 161.
—— 1929. *Anatomy and the Problem of Behaviour*. Cambridge (University Press).
COLE, E. C., 1936. Stain Technology, **11**, 45.
DOHRN, A., 1888. Mitt. zool. Stat. Neapel, **8**, 233.
FREUD, S., 1877. Sitz. Akad. Wiss. Wien, Abth. 3, **75**, 15.
VAN GEHUCHTEN, A., 1895. La Cellule, **11**, 111.
HARDISTY, M. W., 1944. J. anim. Ecol., **13**, 110.
HARRISON, R. G., 1901. Arch. mikr. Anat., **57**, 354.
HATTA, S. J., 1896. J. Coll. Sci. Imp. Univ. Japan, **10**, 225.

HERRICK, C. J., 1908. J. comp. Neurol., **18**, 409.

—— 1944. Ibid., **81**, 307.

HOLMES, W., 1943. Anat. Rec., **86**, 157.

JOHNSTON, J. B., 1902. J. comp. Neurol., **12**, 1.

—— 1905. Morph. Jahrb., **34**, 149.

—— 1910. Anat. Anz., **37**, 153 and 382.

KAPPERS, C. U. A., HUBER, G. C., and CROSBY, E., 1935. *Comparative Anatomy of Nervous System of Vertebrates*. New York (Macmillan).

KOLMER, W., 1905. Anat. Hefte, **29**, 165.

KUPFFER, C., 1890. Arch. mikr. Anat., **35**, 469.

—— 1894. *Studien zur vergleichenden Entwicklungsgeschichte des Kopfes der Kranioten*, 2 Heft. Munich and Leipzig (Lehmann).

—— 1906. *Handbuch der vergleich. u. exper. Entwicklungslehre der Wirbeltiere*, O. Hertwig. Band 2, Teil 3, p. 23. Jena (G. Fischer).

LANG, A. G., 1937. Stain Technology, **12**, 113.

LARSELL, O., 1947. J. comp. Neurol., **86**, 395.

NEAL, H. V., 1897. Anat. Anz., **13**, 441.

—— 1914. J. Morph., **25**, 1.

NONIDEZ, J. F., 1939. Amer. J. Anat., **65**, 361.

PEARSON, A. A., 1936. J. comp. Neurol., **65**, 201.

POLYAK, S. L., 1941. *The Retina*. Chicago (University Press).

SAGEMEHL, M., 1882. Dissertation, Dorpat.

SHIPLEY, A. E., 1887. Quart. J. micr. Sci., **27**, 173.

SMITH, J. E., 1946. Phil. Trans. Roy. Soc., **232**, 279.

STEFANELLI, A., and CAMPOSANO, A., 1946. Pubbl. Staz. Napoli, **20**, 19.

STUDNICKA, F. K., 1895. Sitzungsb. Ges. wiss. Prag, **51**.

TRETJAKOFF, D., 1909. Arch. mikr. Anat., **73**, 607.

—— 1909. Ibid., **74**, 636.

—— 1927. Z. wiss. Zool., **129**, 359.

WALLS, G. L., 1942. *The Vertebrate Eye and its Adaptive Radiation*. Michigan (Cranbrook Inst. of Science).

WINTREBERT, P., 1921. Arch. zool. exp. et gén., **60**, 221.

WOODBURNE, R. T., 1936. J. comp. Neurol., **65**, 403.

YOUNGSTROM, K. A., 1940. Ibid., **73**, 139.

## DESCRIPTION OF PLATES

### PLATE I

A. Transverse section showing the Commissura Infima Halleri. × 1150.

(a) One of the cells of the Commissure.

B. Horizontal section showing several Rohon-Beard neurons with longitudinal and peripheral processes. × 400. Compare Text-figs. 1 and 2.

C. Parasagittal section showing oblique fibre in the lateral part of its course. × 370.

### PLATE II

D. Parasagittal section showing relation of large internuncial cells to the segmental motor nerves and to the intersegmental sensory nerves. × 370. Compare Text-fig. 8.

E. Transverse section, 34μ, showing motor neuron stained with methylene blue. × 1700. Compare Text-fig. 12.

F. Parasagittal section showing a large internuncial cell. × 740.

A

(a)

white

gray

B

dorsal root

Rohon - Beard
neuron

C

anterior

spinal
cord

notochord

WHITING—PLATE I

spinal cord

anterior    D

E    gray    white

dendrite

anterior
dorsal funiculus

F

ventral funiculus

notochord
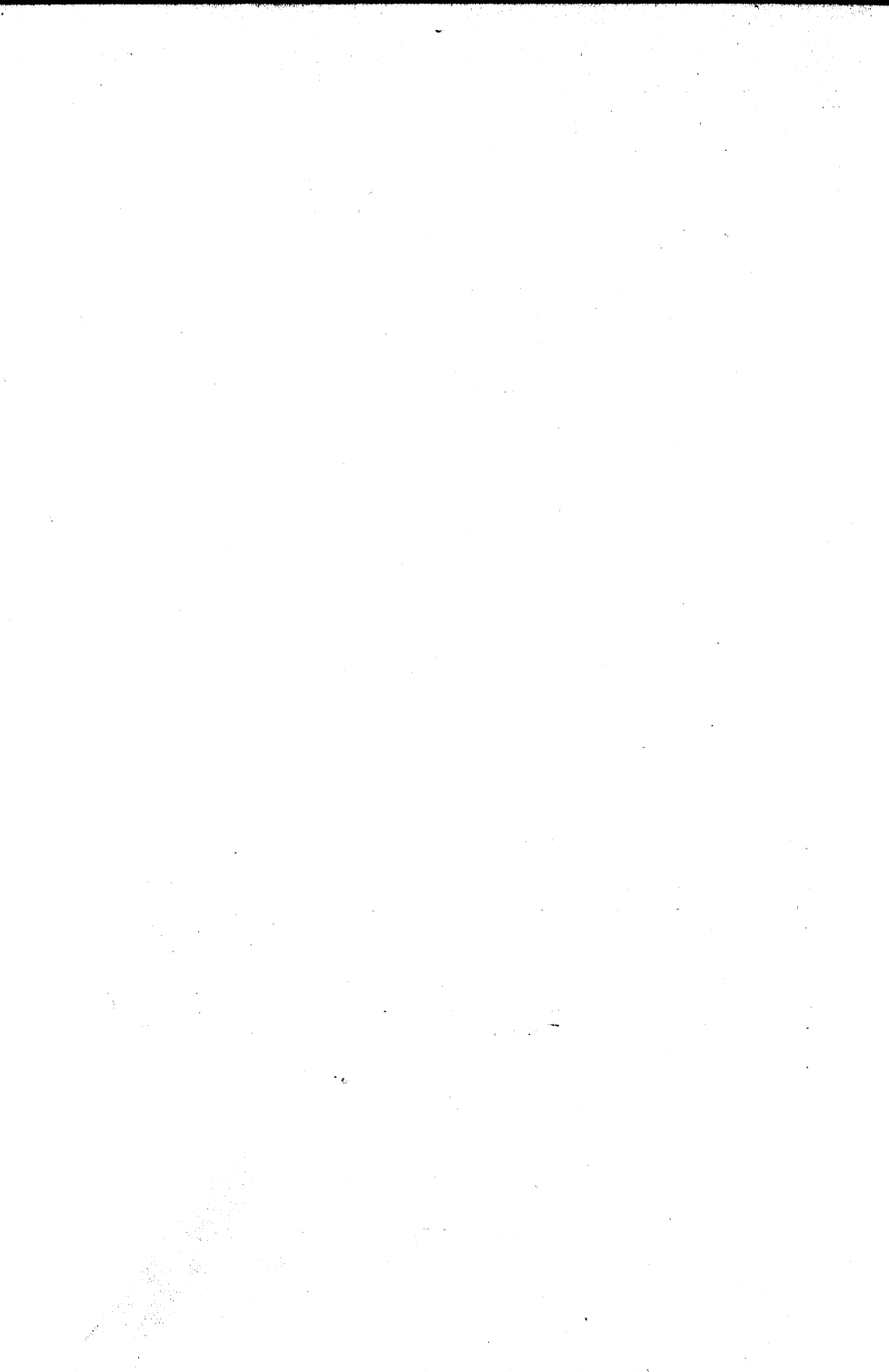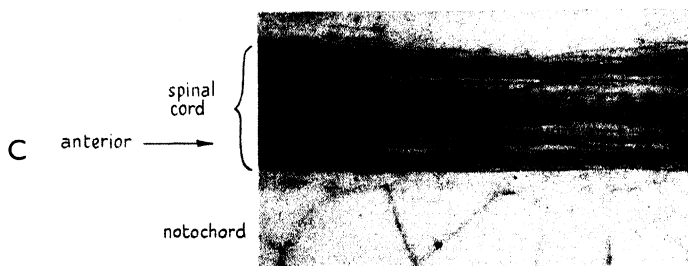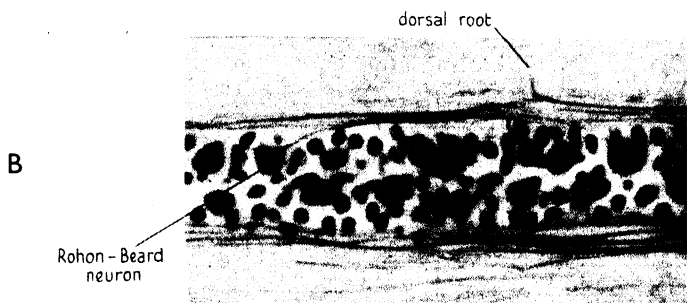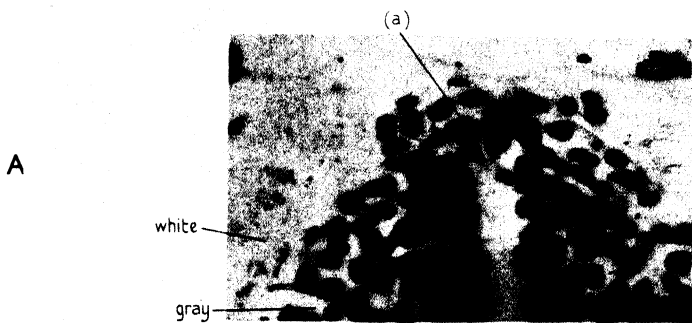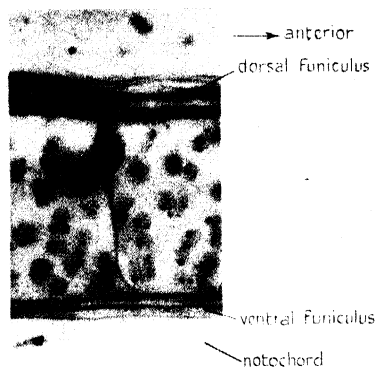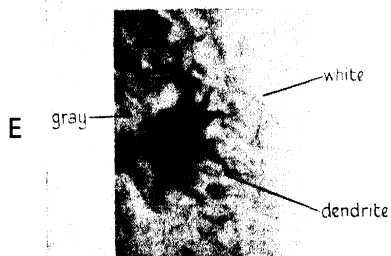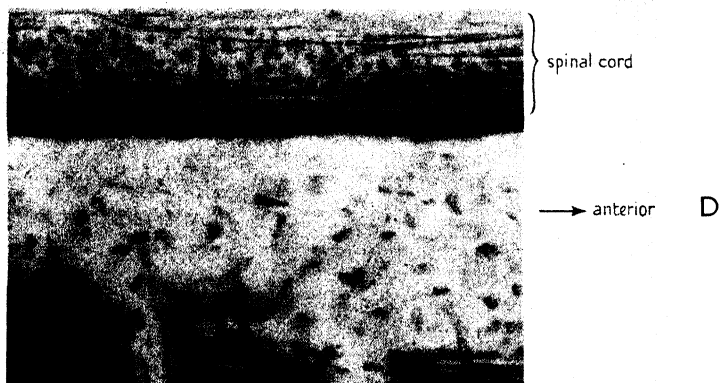
WHITING—PLATE II

# Studies on *Lagenophrys tattersalli* (Ciliata Peritricha, Vorticellinae)

## Part II. Observations on Bionomics, Conjugation, and Apparent Endomixis

### BY

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*(From the Department of Zoology, Victoria University of Manchester)*

### Contents

### INTRODUCTION AND PREVIOUS WORK

THE present paper records the continuation of work on *Lagenophrys tattersalli* Willis, a marine, vorticellinid ciliate found epizoic upon the gill-plates of the amphipod crustacean *Gammarus marinus* Leach (Willis, 1942).

The existence of conjugation ('Copulation', Awerinzew, 1936) in *Lagenophrys* has been known almost from the creation of the genus. Nevertheless, previous accounts of the process are very fragmentary, a result of the acknowledged technical difficulties in studying conjugation in an epizoic, loricate protozoan. In general form, the conjugation of *Lagenophrys* resembles that of other Vorticellinae, and is of the dimorphic type, involving the association of micro- and macroconjugants (cf. Text-fig. 3A). The microconjugant was first observed by Stein (1851). Ubisch (1913) has given the fullest account of the formation of the microconjugant (named by her, variously, as a

'Conjugationsschwarmer' or 'Mikrogamet'). According to this description, based mainly on the freshwater species *L. platei*, the microconjugants are formed by the divisions of an organism produced by *budding* from a normal vegetative individual. Unfortunately the term 'Conjugationsschwarmer' is also applied by Ubisch to this bud. Awerinzew (1936) distinguished two types of microconjugant ('grossen' and 'kleinen' Gameten) in an unnamed, freshwater lagenophryid which was found in West Africa on the crustacean *Telphusa*. These microconjugants are described as being formed from two types of organisms (respectively, the 'grossen' and 'kleinen' Gametocyten) produced by the unequal *fission* of a normal vegetative individual. Since the terminology of Ubisch and Awerinzew is both confusing and inexact, the term 'protoconjugant' is applied in the present work to the organism which divides to form the microconjugants (cf. Text-fig. 2A–C). In the study of *L. tattersalli* special attention has been paid to the formation and subsequent division of the protoconjugant, and a comparison has been made between protoconjugant formation and first-type division (as previously described, Willis, 1942).

In previous work on *Lagenophrys*, little attention has been paid to the nuclear phenomena of conjugation. Awerinzew (1936) alone gives a brief description of the formation of the pronuclei and the synkaryon. Similarly, there is no record of the divisions by which the macronuclear Anlagen, formed from the synkaryon, are distributed among the lineal descendants of the synconjugant (exconjugant). For this reason special attention has been paid to these distributive divisions in *L. tattersalli* where they have an added interest at the period of ecdysis, when they conform to the pattern of a special type of obligatory division (second-type division, Willis, 1942).

There is no record of any endomictic process in *Lagenophrys*, although it may be questioned whether some of the stages figured and described by some earlier workers are not, in fact, stages in endomixis. The difficulty in distinguishing between possible endomixis and conjugation consists in (i) the relatively early fusion of the two partners in conjugation, thus obscuring the distinctive form of the process, and (ii) the fact that the organisms cannot be isolated and cultured away from the host. No attempt has been made in previous work to follow the alternative method of study, namely to trace the behaviour of known individuals upon isolated gill-plates subjected to continual irrigation. The latter method has been employed in the present work.

In conclusion attention must be drawn to certain descriptive terms used in the following account. The surface of the organism applied to the gill-plate, and the free surface, are referred to as the lower and upper surfaces respectively. The diameter passing through the middle of the lorica mouth (oral region) is distinguished as the main diameter, with oral and aboral extremities. The main diameter, together with the diameter at right angles to it, divides up the body into quadrants which are referred to as the right and left oral, and the right and left aboral quadrants.

## METHODS AND MATERIAL

The occurrence and collection of the host and the method for examining living material for prolonged periods by means of Kitching's (1934) irrigation apparatus have been described previously (Willis, 1942).

The difficulties in studying conjugation in *Lagenophrys* may be overcome, in part, by removing the gill-plates from the host, and setting up preparations for continuous irrigation under the microscope. The outline of the gill-plate and the position of the epizoites may then be recorded by means of a camera lucida. In this way the behaviour of conjugants (and other stages) can be followed for periods ranging from 3 to 6 days, and the early phases of conjugation can be distinguished from endomictic or other processes of nuclear reorganization. The sequence of observations on living organisms is interrupted by the first distributive division of the synconjugant, since, as yet, no means has been devised for following the swarmer after its liberation from the parent lorica.

The sea-water used for culturing the hosts, and for the irrigated cultures, was renewed every third day by samples brought directly from the shore at Swanbridge, on the Bristol Channel, where the material was collected.

Material was extracted from the cultures at various stages and fixed in Schaudinn, Champy, or mercuric chloride plus acetic acid for later cytological study.

Since the presence of a lorica made it difficult to obtain an even differentiation, regressive stains, like Heidenhain's iron-alum haematoxylin, were not wholly satisfactory. Diluted Delafield's haematoxylin (1 part stain to 5–10 parts of distilled water), used progressively, gave good pictures of the nucleus, especially in metamorphosing forms. In most cases, however, Feulgen's 'Nuclealfärbung' proved to be the most satisfactory process for demonstrating the nuclear material sharply and evenly. Despite the value of the Feulgen process for nuclear studies, an unqualified reliance upon the method in the study of the complex nuclear phenomena of conjugation does not appear justifiable at the present time. As a result, to assess the value of the method, the Feulgen preparations have been compared, at all critical stages, with preparations made with orthodox nuclear stains. From this comparison, it is clear that the chromatin (basichromatin) is stained energetically by the Feulgen method until the final dissolution of the necrochromidium.

To apply the Feulgen method, the gill-plates were taken, after fixation in mercuric chloride plus acetic acid, and transferred to a little sieve made of cheese-cloth tied around a glass ring (diameter 1 in., depth $\frac{1}{2}$ in.). In this way the preparations can be removed from one fluid to another with the minimum loss of time. This is a great advantage in controlling the critical hydrolysis in N.HCl. Despite the presence of a lorica, the optimum time for the latter is 4 minutes, as in the usual technique for sections of material fixed in mercuric chloride plus acetic acid (Gatenby and Painter, 1937). Some of the preparations were counterstained in light green to give a sharp differentiation of the background.

Estimates of the percentage of the infection transferred from one instar to another of the host were made by taking the moulted cuticles from isolated individuals, and counting the number of empty loricae; the new instar was then killed, after an interval of 3 days, and the new infection counted.

The figures were made with an Abbe camera lucida with a Zeiss oil immersion lens ($\frac{1}{12}$ inch, N.A. 1·30).

## BIONOMICS

Like all species of its genus, *L. tattersalli* is a sessile epizoite. It is restricted to the gill-plates of its host, and occurs most abundantly upon the inner
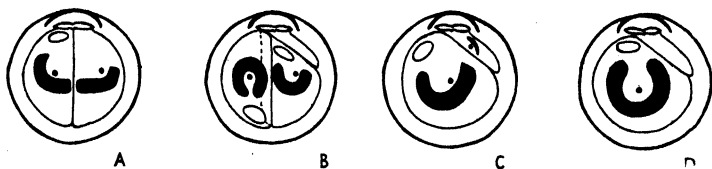


TEXT-FIG. 1. *Lagenophrys tattersalli*. Diagrams illustrating various modes of the divisions occurring between moults and at ecdysis (based on Willis, 1942).

A. 'First-type' division. This normally occurs in the inter-moult period and produces a swarmer (on the left) and a residual organism. The swarmer escapes while the residual organism remains behind in the lorica (thick black outline), grows, and repeats the 'first-type' division process until the ecdysis of the host. B. Mode *a* of 'second-type' division, involving the rapid succession of 'first-' and 'second-type' divisions. The latter is unequal and produces a small residual organism. Two swarmers are thus produced and both escape. The small residual organism remains in the lorica and degenerates. Occurs at ecdysis. C. Mode *b* of 'second-type' division involving the unequal fission of entire organism (i.e. without an immediately preceding first-type division). The micronucleus divides equally, the macronucleus unequally. Occurs at ecdysis. D. Mode *c* of 'second-type' division. The nuclei do not divide. Occurs at ecdysis.

Macronucleus as a broad band (black), micronucleus as a dot (black), peristome as an ovoid outline in swarmer, lorica as a heavy outline. Ciliation omitted.

surfaces of these structures. The reproductive bodies, or swarmers, are 'hypotrichous' forms, adapted for moving over surfaces. They do not appear to become pelagic at any time. From the surface-dwelling habits of the swarmers and the fact that the entire colony is removed periodically by the ecdysis of the host arise two unusual problems for *L. tattersalli*: (i) the problem of maintaining the infection from instar to instar of the host, and (ii) the problem of securing the initial infection of the host. No attention has been paid to these problems in previous work on the genus.

(i) The onset of ecdysis in the host is correlated with the occurrence of second-type divisions of the protozoon (Willis, 1942). Each of these produces a normal swarmer and a small residual body. The various modes of second-type divisions and their relationships to the first-type divisions normally occurring in the inter-moult period are set out diagrammatically in Text·fig. 1. Since all the organisms composing the infection respond to the onset of ecdysis in this way, the whole colony is mobilized when the old cuticle is shed.

The swarmers may then be observed moving freely over the surface of the host. Access to the surface of the underlying new cuticle is provided by splits which appear in the old cuticle before the latter is finally shed. The transference of the infection is not complete, but appears to be about 50–70 per cent. effective. The influence of the obligatory, second-type divisions upon the form of the distributive divisions of the synconjugant is discussed later (*see* Conjugation).

(ii) The initial infection of the host by *L. tattersalli* takes place during the breeding periods of the former. During these periods some of the swarmers emerging from loricae on the gill-plates of female hosts appear to migrate into the brood-pouch which is developed by the latter during breeding periods. If hatching of the eggs contained in the pouch has already occurred, some of the migrating swarmers settle down on the gill-plates of the young gammarids. The brood-pouch is formed by the overlapping oostegites occurring on the anterior thoracic appendages of the female host, and since these oostegites lie close to the heavily infected inner surfaces of the anterior gill-plates of the parent amphipod, a direct pathway is provided for the migration of the swarmers into the brood-pouch. The actual penetration of the swarmers into the pouch has not been observed, but inspection of the gill-plates of young gammarids extracted from the pouch shows that a high proportion are always infected. This initial infection is always low and rarely consists of more than 4–6 specimens of *L. tattersalli* upon a single young gammarid. It follows from this observation, that the colonies of *L. tattersalli* on the gill-plates of a single host are likely to be highly clonic, i.e. to consist of the descendants of the relatively few swarmers which transmit the infection from host to host.

## CONJUGATION

### *Formation and Division of the Protoconjugant*

Conjugation in *L. tattersalli* resembles that of other Vorticellinae and involves the association of two dissimilar conjugants—a larger, non-motile macroconjugant and a smaller, motile microconjugant (Text-fig. 3A). Before the attachment of the microconjugant the macroconjugant resembles a normal vegetative organism in its general morphology, and in the apparent characters of the nucleus. On the other hand, the microconjugant is a specialized organism formed by the binary fission of a protoconjugant (Text-fig. 2A–C).

The protoconjugant is formed by the unequal fission of a vegetative organism (Text-fig. 2A). The plane of cleavage passes through the left oral and aboral quadrants, parallel to the main diameter. In first-type division, cleavage is along the plane of the main diameter.

The first stage in division is the movement of the micronucleus along the anterior border of the macronucleus to a point about two-thirds to three-quarters of the length of the macronucleus from its right extremity. This point marks the position of the future cleavage plane. In first-type division

the micronucleus moves in a similar direction to a point midway along the anterior border of the macronucleus.

The adoral spiral of the parent becomes contracted and the cilia and undulating membranes disappear. Before cleavage is completed structures which resemble the peristome, vestibular cavity, and spiral flange of the early swarmer (Willis, 1942) appear in the protoconjugant (Text-fig. 2A). In the swarmer these structures are formed by the division of the parental peristome, vestibular cavity, and adoral spiral, respectively. There is no con-
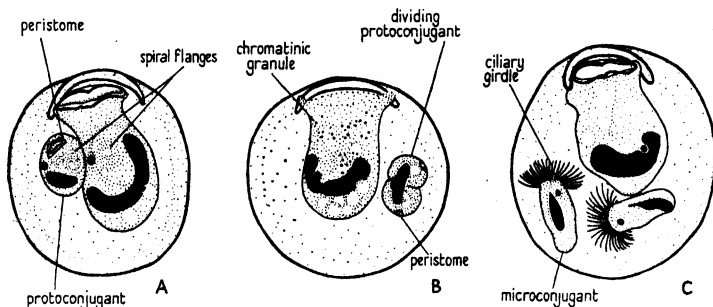


Text-fig. 2. Stages in microconjugant formation. (× 480.) A. Late stage in protoconjugant formation. B. Division of the protoconjugant. From the lower surface. C. Two mature microconjugants within the lorica. Ciliation not visible in the parent organism, and may be absent at this stage. (Feulgen preparations.)

clusive evidence to show whether the peristome, vestibular cavity, and spiral flange of the protoconjugant arise *de novo*, or by division, from the parent.

There is no evidence, in *L. tattersalli*, for the formation of the protoconjugant by budding, as described by Ubisch (1913) for *L. platei*. In the unnamed species described by Awerinzew (1936) the large protoconjugants are formed by a cleavage resembling that which invariably occurs in *L. tattersalli*. On the other hand, the small protoconjugants are said to be formed by a cleavage which runs obliquely across the left aboral quadrant. There is no indication of this type of cleavage in the formation of the protoconjugant in *L. tattersalli*, nor of any dimorphism in size among the protoconjugants. Neither Ubisch (1913) nor Awerinzew (1936) appears to have observed any cytoplasmic differentiation of the protoconjugant which may be compared with that described above for *L. tattersalli*.

At the end of cleavage the protoconjugant separates from the parent organism. At a comparable stage in the development of the swarmer free cilia are formed upon the spiral flange; this never occurs in the protoconjugant. The latter also differs from the mature swarmer in the absence of a sucker cavity and ciliary girdle on the lower surface.

After a short period of free existence the protoconjugant divides within the lorica (Text-fig. 2B) to produce two organisms which transform directly into the functional microconjugants. Before this division the micronucleus takes

up a position midway along the peristomal border of the macronucleus. The cleavage plane passes through this point and the middle of the peristome, and thus, in its general orientation, resembles the cleavage plane of first-type division. In each young microconjugant, a peristome and a small spiral flange are present. Similar structures were not observed by Ubisch (1913) nor by Awerinzew (1936) in the microconjugants of the species studied by them. It is likely that the peristome and the spiral flange are formed by the division of the comparable structures in the protoconjugant, although the evidence for this is not complete.

After the separation of the two young microconjugants, the peristome and the spiral flange disappear in each, and both organisms become elongated (Text-fig. 2c). A ciliary girdle develops around the apical extremity of each microconjugant, in contrast to the swarmer, where the ciliary girdle develops on the lower surface.

It may be concluded from the above account that in *L. tattersalli* the microconjugants are formed by two successive divisions, each of which is comparable, at least in the orientation of the cleavage plane, to first-type division.

### Behaviour of the Microconjugant

After the formation of the ciliary girdle the microconjugants remain within the lorica of the parent for varying periods (in one case for 2 days) before finally escaping. They usually show slow rotating movements. In those cases in which the formation of the microconjugants was traced in life from the protoconjugant, the sequence of observations ended with the escape of the microconjugants through the mouth of the lorica. In many cases, however, the organisms died before escaping. As a result the possibility that some of the microconjugants may fail to escape, and may conjugate with the parental organism, cannot be excluded. In view of the observed escape of some of the microconjugants this conclusion may appear rather fine-drawn. Nevertheless, it is enhanced by the following facts and considerations: (i) The microconjugants are pelagic organisms and are not adapted for moving over surfaces. It is thus difficult to see how they can maintain themselves on the gill-plates while exposed to the respiratory current of the host. It can hardly be supposed that any chemical attraction is exerted upon them by the macroconjugant, since any substance diffusing from the latter would not remain localized. (ii) In a high proportion (about 50 per cent.) of all preparations of early conjugating stages the functional microconjugant is accompanied, in the lorica, by a degenerating body which is clearly comparable, in many cases, to a microconjugant. In view of the above consideration (i), it seems probable that, in such cases as these, the two microconjugants formed from a protoconjugant have been retained within the lorica, and that one has become a functional microconjugant while the other degenerates. (iii) In *L. platei* Ubisch (1913) states that the microconjugant enters the macroconjugant by boring through the lorica. No evidence for this mode of entry has been found

in any living or fixed specimen of *L. tattersalli*. From (i), (ii), and (iii) above it seems reasonable to conclude that the possibility of automictic (paedogamic) conjugation is supported by circumstantial evidence. Among the peritrichous ciliates, paedogamic conjugation has been observed by Enriquez (1907) in *Opercularia*.

### Nuclear Phenomena in the Micro- and Macroconjugants

In living material the first sign of impending conjugation is the rapid darting movement of the microconjugant towards the surface of the left
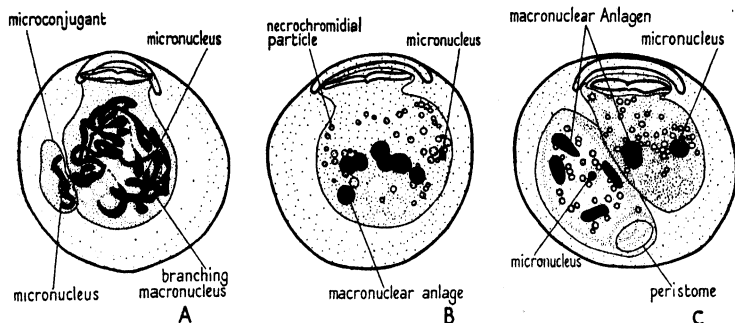


TEXT-FIG. 3. Stages in conjugation up to the first distributive division. Ciliation omitted. (×480.)

A. Early stage in the association of the conjugants. The macronucleus of the macroconjugant has the branching form which is developed shortly before its fragmentation. B. Mature synconjugant. The micro- and macroconjugants have fused. C. Appearance of the first distributive division in the inter-moult period. (Feulgen preparations.)

aboral quadrant of the macroconjugant. Although there is no visible differentiation of the macroconjugant in this area, the fact that the movements of the microconjugant are always directed towards it leads to the supposition that it must exert some attraction. It is physically possible for the microconjugant to attack any point on the surface of the macroconjugant. The movements of the microconjugant are brought about by the concerted, backward sweep of its cilia.

The microconjugant eventually becomes attached to the macroconjugant by its apex, and not by its side. This is in contrast to the behaviour of the smaller organism produced in apparent endomixis (*see* Observations on Apparent Endomixis). The cilia of the microconjugant later disappear.

The cytological study of specimens at this early stage shows that there are no preparatory nuclear changes in either conjugant. This observation is of some importance, since Ubisch (1913) concluded that in *L. platei* there were preparatory nuclear changes in the macroconjugant, before the union of the latter with the microconjugant. The evidence for this conclusion is criticized later (*see* Metamorphosis and the Later Distributive Divisions).

After the disappearance of the cilia from the microconjugant the protoplasm of both conjugants becomes confluent at the point of attachment. The

micronucleus of each conjugant moves towards the point of confluence and undergoes a series of divisions. The divisions are synchronous in both conjugants, and are exactly comparable to those observed by Awerinzew (1936) in *Lagenophrys* sp. and to the maturation divisions of other Vorticellinae. In *L. tattersalli*, during the maturation divisions, the chromatin appears finely dispersed through the substance of the nuclei and no chromosome-like structures are visible. After the exchange of the migratory pronuclei, a synkaryon is formed in the macroconjugant. No synkaryon was observed in the microconjugant. The nuclear behaviour of the two conjugants ceases to be synchronous after the exchange of the pronuclei. In the microconjugant the latter quickly degenerate and disappear. This degeneration is accompanied by the absorption of the microconjugant by the macroconjugant. In view of this it seems preferable to describe the macroconjugant, subsequently, as a syn- rather than ex-conjugant. Further, in view of the fusion of the conjugants and the formation of only one functional synkaryon there is some justification for describing the sexual process of *Lagenophrys* as copulation (as defined by Doflein, 1929), rather than as conjugation.

After the attachment of the conjugants the macronucleus of the macroconjugant undergoes a remarkable transformation which has not been observed in any other species of *Lagenophrys*, and which has no exact counterpart in other ciliates (Text-fig. 3A). The macronucleus throws out branching processes which appear to become drawn around in the cytoplasm. In this process parts of the branches become attenuated and finally break, so that large separated fragments of the macronucleus become dispersed throughout the cytoplasm. At this stage there occurs a further fragmentation of the macronuclear substance and, as in conjugation and endomixis in other ciliates, an extensive necrochromidium is formed. This consists of a large number of small vesicles, each with a cortex of chromatin which surrounds a clear space.

### The Maturation and the First Distributive Division of the Synconjugant

After syngamy the synkaryon moves into a central position and undergoes three successive divisions to form eight nuclei (as in *Vorticella*, Maupas, 1888, and *Carchesium*, Popoff, 1908). Seven of these become macronuclear Anlagen, one forms a micronuclear Anlage. Before dividing the synkaryon increases in size and becomes a somewhat ovoid vesicle. In Feulgen preparations, this is seen to consist of a pale-staining matrix of finely dispersed chromatin containing larger granules which stain intensely.

In most cases the divisions of the synkaryon occur in rapid succession and are synchronous. Therefore, in later stages of maturation, when the chromatin content of the Anlagen increases, the latter differentiate uniformly. The micronuclear Anlage later decreases in size to the dimensions of a normal micronucleus and its chromatin becomes condensed, the entire Anlage appearing progressively more homogeneous and deeply staining after Feulgen. In the mature macronuclear Anlagen the chromatin appears coarsely granular

and is arranged in a network. The general appearance of the mature syncon-
jugant is shown by Text-fig. 3B. The macronuclear Anlagen and the micro-
nucleus usually take up a position which roughly corresponds to the positions
of a macro- and micronucleus in a normal, vegetative organism.

In certain cases the successive divisions of the synkaryon appear to occur
less rapidly and without synchronization. This condition is indicated by
the small number of maturing synconjugants in which the members of
each set of macronuclear Anlagen show variation in size, and in chromatin
concentration.

Between moults the first distributive division of the mature synconjugant
is always a first-type division which is normal in all but the condition of the
macronuclear material. The micronucleus moves into a central position on
the main diameter and determines the position of the future cleavage. The
seven macronuclear Anlagen become segregated into groups of three and four.
Usually the group of four Anlagen passes to the swarmer (Text-fig. 3C), the
remainder being retained in the residual organism. The lipoid and fatty
reserve materials are either divided equally or segregated in the swarmer,
as in normal, non-distributive first-type divisions (Willis, 1942). The food
vacuoles are retained in the residual organism while the necrochromidium is
divided equally between the swarmer and the residual organism. Since all
these inclusions (i.e. fat, lipoid, food vacuoles, and necrochromidium) are
uniformly dispersed throughout the cytoplasm before division, it is clear that
simple cleavage cannot account for the independent segregations which have
been observed. As yet no explanation can be given of this feature of the
division process. At the end of cleavage the macronuclear Anlagen of the
swarmer become elongated. This change of form seems to be comparable to
the elongation and attenuation of the macronucleus in the non-distributive
swarmer of asexual reproduction (Willis, ibid.).

At ecdysis the obligatory second-type division cuts across the normal
progress of maturation, and may considerably modify the character of the
first distributive division. In consequence conditions are often observed
which appear to have no parallel among the free-living ciliates. A comparison
with the behaviour of other ciliates which are epizoic upon arthropods would
be of interest, but this is impracticable owing to the lack of previous work on
the responses of the epizoites to the ecdyses of their hosts.

The behaviour of the synconjugant at ecdysis may be considered in two
categories, (i) when the synconjugant is mature at ecdysis, and (ii) when it is
immature at ecdysis.

(i) The mature synconjugant has been found to respond to ecdysis by
modes *a* and *c* of second-type division.

In mode *a* responses (i.e. when there is a rapid succession of first- and
second-type divisions) the first-type division is usually comparable to that
which normally occurs in the inter-moult period. The following second-type
division then produces a swarmer with three Anlagen, and a small residual
organism.

In mode *c* responses (Text-fig. 4B) the seven macronuclear Anlagen become grouped in a circle which occupies a position similar to that occupied by the horseshoe-shaped macronucleus of an asexual swarmer. The micronucleus lies centrally, within the macronuclear Anlagen. The latter are ovoid in the distributive swarmer. The small residual organism contains a small portion of the necrochromidium which is cut off from the main mass by the cleavage plane.

(ii) In cases where the synconjugant is immature at ecdysis the second-type divisions may again be by modes *a* and *c*. The first-type division of an
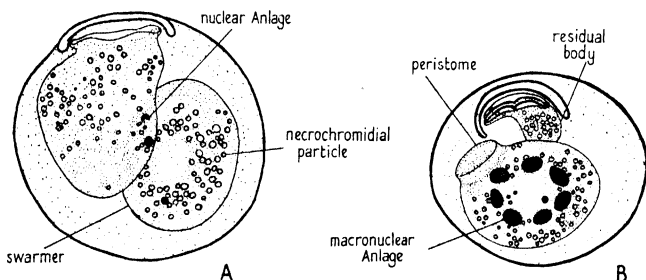


TEXT-FIG. 4. Stages in second-type division at ecdysis, showing modifications of the first distributive division. Ciliation omitted. (×480.)
A. First-type division of a mode *a* response in which one of the divisions of the synkaryon appears to have been cytoclastic. From the lower surface. B. Mode *c* response. (Feulgen preparations.)

incompletely developed mode *a* response is shown by Text-fig. 4A. The swarmer possesses one condensed nuclear Anlage, while two similar bodies are present in the residual organism. These nuclear structures are smaller than the macronuclear Anlagen of a maturing synconjugant. They resemble normal micronuclei in size, but contain less chromatin. In this specimen it seems clear that one of the divisions of the synkaryon has become *cytoclastic*, and has determined the equal, binary fission of the synconjugant. Unfortunately it has not been possible to trace the later behaviour of the swarmers produced by this unusual type of distributive division. It seems reasonable to suppose that the nuclei which are present in such swarmers (and those of the residual organism as well) later divide further, as if they were part of the normal series of nuclei produced by the synkaryon during the intermoult period.

## Metamorphosis and the Later Distributive Divisions

Owing to the impossibility of following the path of the swarmer, the lineal descendants of the marked forms kept under observation up to the period of the first distributive division could not be traced. The following account is therefore based upon the seriation of various fixed stages.

The metamorphosis of forms with four Anlagen has been observed frequently (Text-fig. 5A, B). The figured specimens show the metamorphosis of swarmers produced by the first distributive division. The reorganization of the nuclear material produced by conjugation is without effect on the complex process of metamorphosis (as previously described, Willis, 1942), except that (i) the micronucleus may take up various positions, in contrast to normal metamorphosis, when it lies embedded in the right limb of the macronucleus, and (ii) the greatly distended growth cavity appears to exert less pressure upon the scattered macronuclear Anlagen than upon the normal macronucleus, which becomes greatly attenuated during metamorphosis (Willis, 1942).
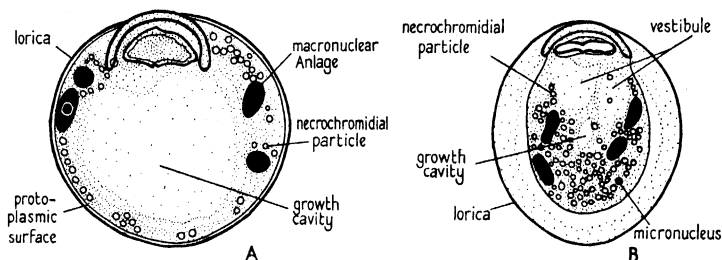


TEXT-FIG. 5. Early (A) and late (B) stages in the metamorphosis of swarmers formed by the first distributive division. Ciliation omitted from B. (×480.) (Delafield's Haematoxylin, after Schaudinn fixation.)

In vorticellinids generally, three generations are required to distribute the macronuclear Anlagen of the synconjugant. As a result eight organisms are produced, each with a single macro- and micronucleus. In a number of cases, however, forms are found in which the morphologically single macronucleus shows a gross, moniliform character, with two, three, four, or even seven lobes, i.e. with the number of lobes corresponding to the numbers of discrete Anlagen found in normal distributive stages. This may indicate that in certain cases the Anlagen become fused together. If this is so, then certain apparently normal asexual divisions may in fact be distributive.

The necrochromidium persists throughout the series of distributive divisions and, in some cases, even later. The constituent particles retain their vesicular character.

In *L. platei* Ubisch (1913, Text-fig. 44) describes and figures certain specimens which bear a close resemblance to some of the distributive generations of *L. tattersalli*. Ubisch, however, did not consider the distributive divisions, and interpreted these specimens as stages in the maturation of the macroconjugant in readiness for conjugation (Conjugationserwartung). In view of the absence, in *L. tattersalli*, of nuclear preparation in the macroconjugant before its union with the microconjugant, it seems highly probable that the 'Conjugationserwartung' of Ubisch is based upon specimens produced by the distributive divisions of a synconjugant.

*The Normal Process*

The analysis of colonies showing 'epidemic' conjugation always reveals a small number of forms which, to a superficial view, appear to be undergoing conjugation (Text-fig. 6A, B), but which, on closer inspection, show certain differences from the normal mode of that process.

These forms appear to consist of two unequal organisms which never separate. The smaller organism shows some resemblance to a protoconjugant which has failed to separate from its parent. It differs from a protoconjugant in the following characters: (i) the cleavage plane, by which it is formed, runs
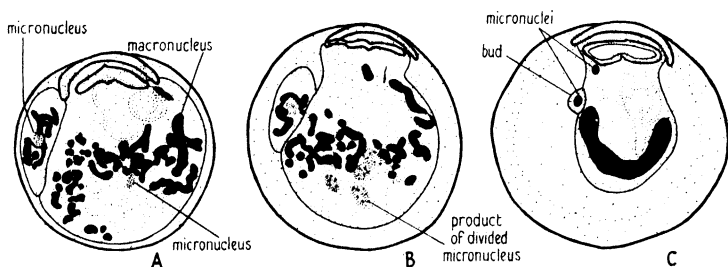


TEXT-FIG. 6. A. Early stage in apparent endomixis, showing the formation of the larger and smaller organisms (the smaller organism is on the left-hand side). B. Later stage in apparent endomixis. The micronucleus of the smaller organism is undivided; the micronucleus of the larger organism has undergone two successive divisions. C. Late stage in a process of anomalous micronuclear division. Ciliation omitted. All figures ×480. (Feulgen preparations.)

obliquely across the left oral quadrant of the parent, and (ii) the peristome, vestibular cavity and dexiotropic spiral flange of the typical protoconjugant are absent. The smaller organism differs from a microconjugant in that (i) it is not formed by the binary fission of a protoconjugant, and (ii) it is attached along the whole of its side to the left oral quadrant of the larger organism, and not by its apex to the left aboral quadrant, as in the case of the attachment of the micro- to the macroconjugant in conjugation.

Morphologically, the smaller organism may be regarded as a protoconjugant which has failed to separate from the parent, and which has undergone no further differentiation.

Differences from the process of conjugation are also shown by the behaviour of the nucleus in the larger and smaller organisms. In the first place, the micronucleus of the smaller organism, although it becomes enlarged and vesicular as in the first stages of the maturation of the micronucleus of the microconjugant, does not develop further, and appears to degenerate without division. In the larger organism, the micronucleus takes up a central position. In a macroconjugant, on the other hand, the micronucleus becomes eccentric and moves towards the area of confluence with the microconjugant. Later, the micronucleus of the larger organism becomes enlarged and vesicular, and

divides twice to form four daughter nuclei (Text-fig. 6B). There is no evidence that a third division occurs, as in the case of the dividing synkaryon formed in conjugation. Further differences from the nuclear phenomena of conjugation are also shown in the formation of the necrochromidium. Thus, the filaments produced from the macronucleus are always short and lie, like the vesicles to which they eventually give rise, along the original traverse of the unfragmented macronucleus. The behaviour of the larger organism was not traced beyond the stage represented by Text-fig. 6B. This circumstance may, of course, be owing to a lack of material. On the other hand, it is quite possible that in the later stages the distinguishing morphological feature of the process is obscured by the fusion of the larger and smaller organisms. On the assumption that three of the four nuclei which are formed by the division of the micronucleus in the larger organism become macronuclear Anlagen, the product of the fusion of the larger and smaller organisms would then be indistinguishable from a post-conjugation distributive form with three macronuclear Anlagen.

It is clear from the above that the process under consideration in this section must be sharply distinguished from normal conjugation. From the information available it may be described as an unusual, endomictic reorganization of the nuclear apparatus.

### An Anomalous Process of Micronuclear Reduction

At various periods specimens of *L. tattersalli* are found with minute buds attached to the surface of the left oral quadrant, opposite to the left extremity of the macronucleus (Text-fig. 6C). These buds consist of a small mass of protoplasm surrounding a rounded inclusion which resembles a normal micronucleus in size, form, and reaction (to Feulgen and other nuclear stains). This inclusion is undoubtedly a nucleus which is produced by the fission of the micronucleus of the larger organism, to which the bud is attached. This conclusion is based on the discovery of the simple intermediary stages in bud formation. In the first place the parental micronucleus leaves its bay on the right-hand side of the macronucleus, moves along the anterior border of the latter, and eventually reaches a position below the protoplasmic surface in the area where the bud is later formed. The micronucleus divides into two at this point. One of the daughter nuclei, with a small quantity of undifferentiated protoplasm, forms the bud. Whether the latter is formed by true budding—i.e. by an outflow of the protoplasmic surface—or by fission has not been ascertained. The macronucleus remains unmodified throughout the process.

Buds of a similar type, and up to seven in number in a single lorica, were observed by Ubisch (1913) in *L. platei*. Ubisch regarded bud-formation as a process of micronuclear reduction which preceded conjugation. The evidence for this conclusion seems negligible, and to be based on specimens in which the buds occur, in the same lorica, together with a larger vegetative organism which possesses a fragmented macronucleus. In the larger organism,

however, the figures (ibid.) show clearly that the pattern of the nuclear material resembles that of a distributive generation in *L. tattersalli*. It seems likely, therefore, that Ubisch was led into error through failing to recognize the distributive divisions of the synconjugant.

In *L. tattersalli* the buds are never found in association with conjugants or in any constant relationship to a distinctive phase of the life-history. At present the point of importance appears to be that these buds are actually formed by the organism to which they are attached, and that they are not foreign bodies or degenerated microconjugants. The recognition of this fact has not previously been possible owing to the lack of observations on the intermediary stages of bud-formation.

### SUMMARY

1. A study of the bionomics of *L. tattersalli* shows that the initial infection of the host is by the passage of swarmers from the gill-plates of the female host to those of the embryo in the brood-pouch. The transmission of the infection from instar to instar is about 50–70 per cent. effective. It is brought about by (i) the mobilization of the entire colony at ecdysis by an obligatory type of division (second-type division), and (ii) by the penetration of the swarmers to the surface of the new instar through splits which appear in the old cuticle as it is shed.

2. The term 'protoconjugant' is applied to the organism from which the two microconjugants are formed by binary fission. The protoconjugant is formed by the unequal fission of the parent, and not by budding as in *L. platei* (Ubisch, 1913). The divisions which produce the proto- and microconjugants are compared with the normal asexual reproductive process (first-type division, Willis, 1942).

3. The conjugation process is described. In view of the early fusion of both conjugants, the term 'synconjugant' is proposed for the macroconjugant after its fusion with the microconjugant. There are no visible nuclear preparations for conjugation in either conjugant before their attachment. The evidence for a state of 'Conjugationserwartung' (Ubisch, 1913) is criticized and shown to be based, in all probability, on specimens produced by the distributive divisions of the synconjugant.

4. The term 'necrochromidium' is proposed for the mass of vesicles formed by the degeneration of the macronucleus in conjugation and endomixis. Before the formation of the necrochromidium the macronucleus of the macroconjugant becomes elaborately branched.

5. The distributive divisions of the synconjugant are described for the first time. Resemblance to other vorticellinids is shown during the intermoult period of the host. At ecdysis, the distribution of the nuclear Anlagen is adapted to the obligatory second-type divisions which are undergone by *L. tattersalli* at this period. In some cases one of the divisions of the synkaryon is cytoclastic.

6. A process of apparent endomixis is described. In this process unequal fission produces a small organism, resembling a protoconjugant which fails to separate, and a larger organism. The micronucleus of the smaller organism remains undivided and soon degenerates. The micronucleus of the larger organism undergoes two successive divisions to form four nuclear Anlagen.

7. An anomalous process of micronuclear division and reduction is described. One of the micronuclei produced by the division passes into a small bud of protoplasm. Later the latter falls off and degenerates.

## REFERENCES

AWERINZEW, S. W., 1936. Archiv. für Protistenk., **87**, 131.
DOFLEIN, F., 1929. *Lehrbuch der Protozoenkunde.* Jena (Gustav Fischer).
ENRIQUEZ, P., 1907. Archiv. für Protistenk., **9**, 195.
GATENBY, J. B., and PAINTER, T., 1937. *Microtomist's Vade-Mecum.* London (Churchill).
KITCHING, J. A., 1934. Journ. exp. Biol., **9**, 364.
MAUPAS, E., 1888. Arch. Zool. exp. et gén., **6**, 165.
POPOFF, M., 1908. Z. wiss. Zool., **89**, 478.
STEIN, F., 1851. Ibid., **3**, 475.
UBISCH, M., 1913. Archiv. für Protistenk., **29**, 39.
WILLIS, A. G., 1942. Quart. J. micr. Sci., **83**, 171.

# The Golgi Material and Mitochondria in the Salivary Glands of the Larva of *Drosophila melanogaster*

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## INTRODUCTION

THIS paper is the second report on a study of the cytoplasmic element in some of the larval tissues of *Drosophila*. As with the previous study (1947), this one lays emphasis on the morphology, distribution, and behaviour of the cytoplasmic elements in the cells.

## MATERIAL AND METHODS

For this study 'wild' flies of *Drosophila melanogaster* were used. The cultural conditions under which the larvae were raised were the same as those under which material was obtained for the previous study. Preliminary observations made on some slides convinced me that in order to understand some of the phenomena observed, it would be necessary to study glands from larvae of many different stages of development. Material was therefore prepared with each one of the many fixatives and stains employed in this study from larvae of about 2, 3, 4, and 5 mm. long, and also from those which had already become quiescent for 3 or 4 hours. These, of course, are arbitrary stages and do not have any developmental basis; but as a means of securing material involving a time factor the method has proved of service for my purpose. Fixatives and stains used were the standard ones for demonstrating Golgi material and mitochondria, such as Champy–Kull, Kolatchev, Mann–Kopsch, Benda as modified by Baker, &c. The last-mentioned technique has given me very satisfactory slides for mitochondria, while Mann–Kopsch slides have proved to be most useful for Golgi material observations. Mann–Kopsch material counter-stained according to Altmann, when once the correct dura-tion of the baths in potassium permanganate and sulphurous acid was found, has given me brilliant differential staining of both categories of elements in the same cell.

From the standpoint of structure and normal development, the salivary glands of *Drosophila* larvae have been investigated by Makino (1938), Ross (1939), Sonnenblick (1940), and Bodenstein (1943). This report deals only with the two cytoplasmic constituents in the cells of the glands at various stages of development.

### OBSERVATIONS

Before presenting my observations it will be helpful to give as a background some facts about *Drosophila* larva salivary glands discovered by the last two investigators mentioned above. Sonnenblick (1940) showed that the increase in size of the glands is due to the growth of the component cells in the plates which initially invaginate to form the glands; the number of these cells remains constant throughout the development of the glands. Bodenstein (1943) in his study of the normal development of the salivary glands reported the following facts which are of interest in connexion with this report: (1) up to the second moult the growth-rate of all the cells is apparently uniform throughout the gland; (2) from the second moult on, however, the cells in a gland do not all grow at the same rate, those in the distal portion of the gland exhibiting a higher rate of growth and consequently being bigger than the more proximally located ones; (3) though at certain stages of development the difference in size between the cells at the proximal end and those at the distal end is quite pronounced, the increase in diameter of a gland in the proximal-distal direction is a gradual one. At no time in the larval life of a *Drosophila* do its salivary glands show a sharp separation into a proximal and a distal portion. 'The form and size of the salivary gland seem thus to be determined by two different growth rates in two different directions: one correlated with age affects the gland as a whole, and the other constitutes a proximal-distal gradient which determines the size of the cells throughout the length of the gland' (Bodenstein, 1943).

My observations, though made with other aims in mind, confirm the facts listed above except the point concerning the number of cells with which a *Drosophila* larva salivary gland begins and ends its development. The nature of my material does not lend itself readily to such a study. This list of facts, so far as they concern this report, may be summed up as follows: the cells throughout a gland from a larva before the second moult are practically all in the same stage of development. This is also true of practically all the cells in a gland of a fully developed larva. However, the cells in the glands of larvae at any stage of development between these two differ among themselves in their degree of maturity according to their location on the long axis of the gland, the more distally situated cells being bigger and more mature. Cells of one stage of maturity found at one locus in the gland of a larva at a certain stage of development are also found at a more proximal position in the gland of a larva more advanced in age. This having been ascertained for a fact, I have found it both simple and instructive, while making my observations, to concentrate on following the changes which the cytoplasmic constituents undergo in cells of different degrees of maturity rather than paying too much attention to the particular stage of development of a gland as a whole. The matter is thus reduced to placing in order the changes observed in the Golgi material and mitochondria as they occur in cells from very young ones to those which have begun to exhibit cytolysis.

*Golgi material.* In the cells of the youngest material I have prepared, the Golgi material occurs in discrete particles, mostly of an irregular shape, though some may appear roundish, but never of a smooth outline such as chondriomites assume (Text-fig. I. 1). At this stage they appear homogeneous, but occasionally in one or two of them a light area is faintly observable. The number of individual elements in each cell as seen in sections of $5\mu$ is around twelve. Their distribution in the cell is apparently a haphazard one: they do not seem to favour any particular location in the cell, such as the neighbourhood of the nucleus, the basal pole or the lumen pole region, &c. Besides the Golgi elements very minute droplets or vacuoles of about the same size as the Golgi elements can be seen lying free in the cytoplasm. Since they are not stained and are very small they are not at all conspicuous; but they are unmistakably visible once an observer is made aware of their presence.

In older cells such as depicted in Text-fig. I. 2 and 3 the Golgi bodies have increased in size accompanying a size increase in the cells themselves, though their number seems to have remained about the same as in the cells of the previous stage. The lighter area in some Golgi elements is now easily visible, and the droplets lying free in the cytoplasm are also bigger than those found in the younger cells. The presence of a number of relatively larger vacuoles besides the usual small droplets in the cell as shown in Text-fig. I. 3 is interesting in connexion with what Baptist (1941) discovered in the salivary glands of Hemiptera-Heteroptera. The minute droplets lying free in the cytoplasm do not seem to have a nuclear origin so far as observable evidences indicate. Neither do slides made to demonstrate mitochondria point to a mitochondrial origin for them. Having worked on the Golgi material in *Drosophila* larvae for some time, one is tempted to regard these small spherical droplets as products elaborated by the Golgi elements and set free in the cytoplasm, the bigger vacuoles being simply the result of fusion of two or more of the minute droplets. This idea is amply borne out in cells of the next stages of development (Text-fig. I. 4, 5, 6, and 7). These cells are larger and the individual Golgi bodies have also increased in size. They are thus much more favourable material for critical study. The condition found in cells in these stages as regards the Golgi bodies is such as to duplicate that found in the actively secreting cells of the glandular portion of the proventriculus of the larva (1947). Text-fig. I. 6 shows a cell in which two or three of the secretion droplets are about to be entirely liberated from their respective Golgi shell. The only difference between the two cases is that in the proventriculus the droplets released from the Golgi material do not fuse to form bigger vacuoles as they do in the present case.

All the four figures referred to above show the cells with their lumen ends broken, releasing into the lumen a portion of their cytoplasm together with the secretion vacuoles and droplets. So, it seems, there is on the part of the cells a periodic release of the digestive enzyme or enzymes elaborated by the Golgi bodies. But the cell itself is never wholly destroyed. On the basis of the absence of mitosis and of replacement cells, and of the fact that no
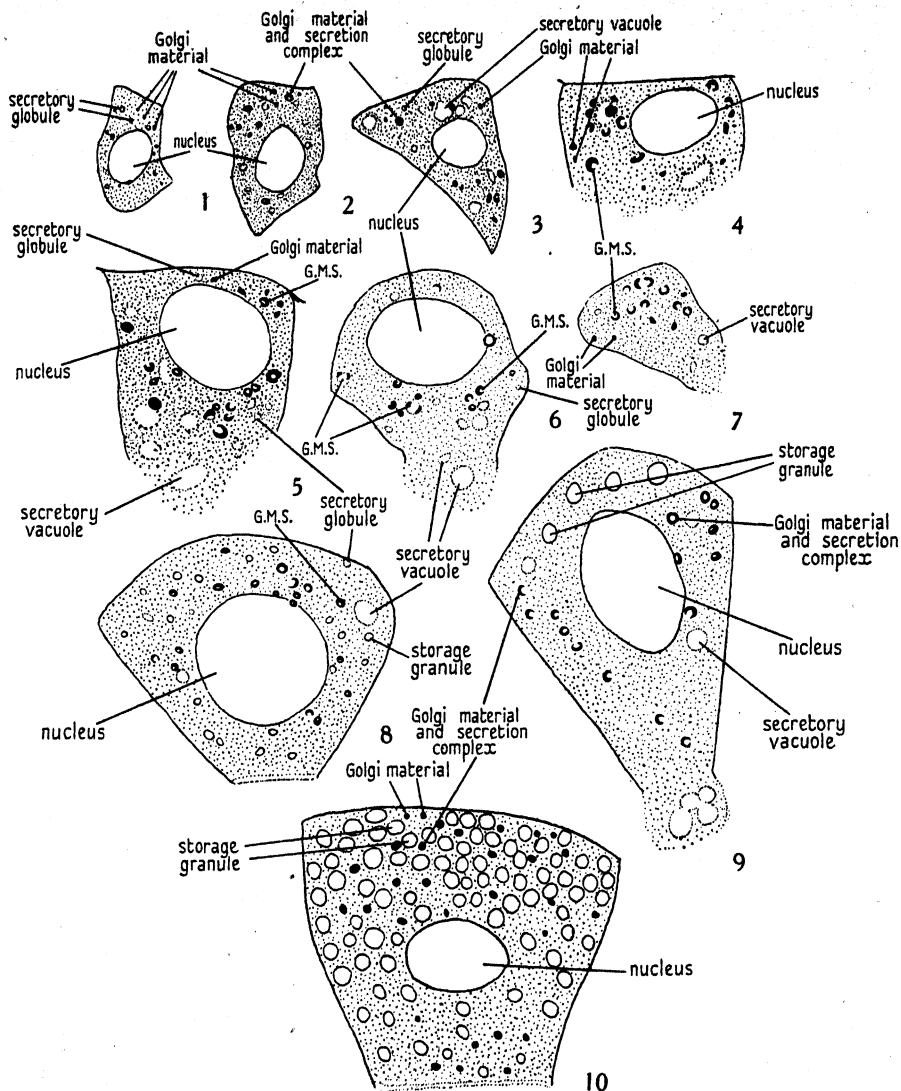
TEXT-FIG. I

All figures are camera-lucida drawings made at ×1,150 the originals, except Text-fig. IV. 26, which is ×510. G.M.S.=Golgi material and secretion complex.

1. A very young cell showing homogeneous pieces of Golgi material in one of which a light centre is barely visible; secretory globules of about the same size as the larger Golgi elements are seen lying free in the cytoplasm. Mann–Kopsch.

2. An older cell than the one shown in Fig. 1; Golgi elements have increased in size and the secretory globules contained within them are more easily discernible; free secretory globules have also increased in size and number. Mann–Kopsch.

nucleus of any cell has been seen to be affected in the process of releasing by the cell of its secretory material, it must be concluded that the cells have the power to repair themselves after the function of discharging the secretion into the lumen has been fulfilled. In this connexion it is interesting to note that cells with a broken inner wall and gland lumens partially filled with secretion are more often observed in younger glands (Text-fig. II. 14) than in those having reached a certain stage of maturity. I shall, however, return to this point in a later paragraph.

In the next stage of growth a cell shows its Golgi elements in just about the same condition (Text-fig. I. 8). But besides the Golgi bodies and their released droplets, both coalesced and uncoalesced, granules of a new type are to be found. These granules have at this time about the same size as the Golgi bodies, but appear to possess a less watery consistency than the digestive droplets. In material osmicated correctly for critical Golgi material study they appear faintly grey. A cell of the next advanced stage shows these granules bigger and more numerous. In such a cell the Golgi bodies present a picture not much different from what can be seen in cells one stage younger. Text-fig. I. 9 shows a few large granules besides the Golgi bodies and their released secretion. This figure is interesting because some of its fused drops of digestive secretion are going through further coalescence in the mass of cytoplasm which has already broken into the lumen of the gland.

From this stage on and so far as the Golgi material is concerned the condition remains about the same, except that as the cells grow bigger it becomes increasingly difficult to observe the droplets and vacuoles. But Text-figs. I. 10, and II. 11 and 12 show the sequence of events in the development of the granules, leading eventually to a stage wherein a cell may be said to be literally filled with them. These figures represent cells in which the Golgi bodies are a little over-impregnated, as a result of which the drop of secretion contained in each Golgi element does not stand out so clearly. It will also be noticed in Text-fig. I. 10 that there are more of these granules at the basal end of the cell—a condition very often observed. This seems to suggest that the origin and growth of these granules begin from the basal pole of the cell. Text-fig. II. 12 shows a cell with its full load of granules already developed. In fact, it is evident that its lumen pole has begun to show signs of disintegration as indicated by a layer of very darkly stained cytoplasm.

3. A still older cell in which vacuoles of secretion are formed as a result of fusion of two or more individual secretory globules. Mann–Kopsch.

4–7. Four cells of increasing degree of maturity showing changes undergone by Golgi elements and their spatial relation to the secretory globules. Fig. 6 illustrates the setting free of the secretion droplets as a result of the breaking of their respective confining Golgi shell. The cells are depicted with their lumen end broken, releasing into the gland lumen vacuoles of secretion and a part of cytoplasm. Mann–Kopsch.

8–9. Two cells of different stages of development showing the appearance of the storage granules in the cytoplasm. Mann–Kopsch.

10. A cell of advanced maturity showing the storage granules increased in size and number. Kolatchev.
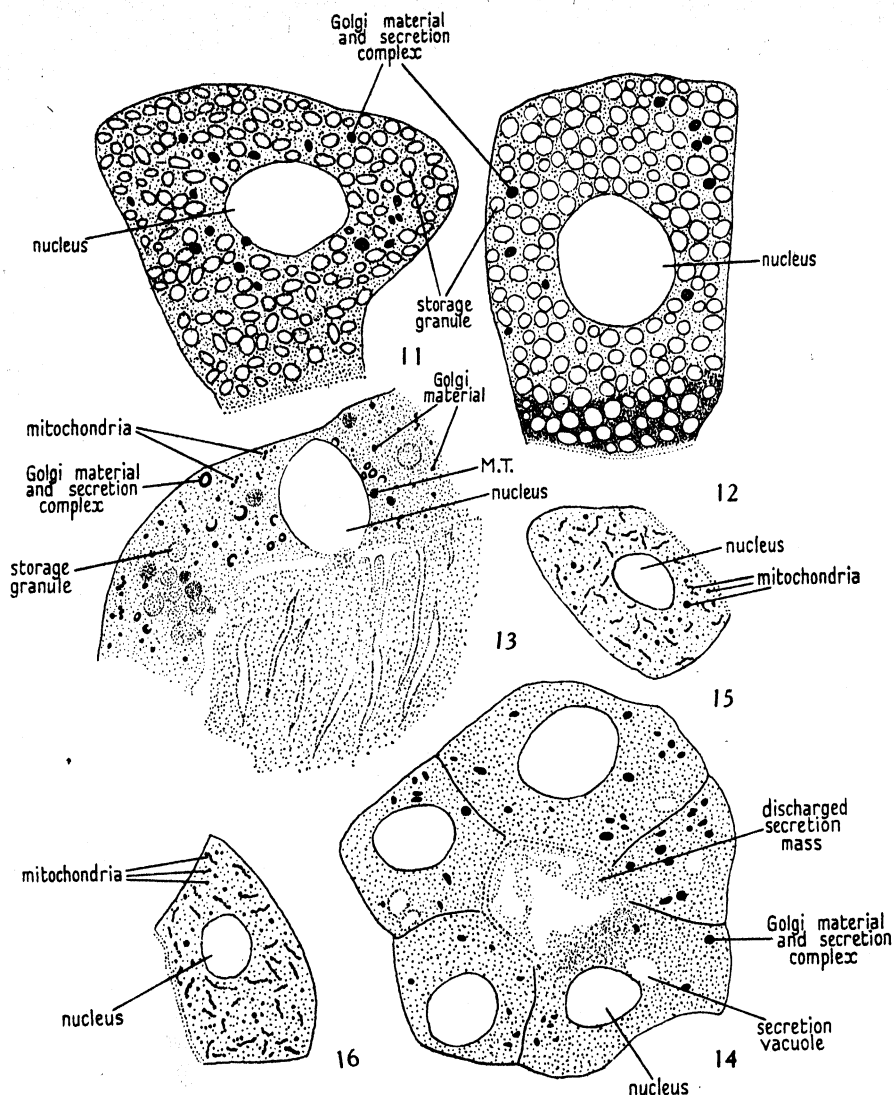
TEXT-FIG. II

11–12. Two mature cells full of storage granules. Fig. 12 shows the cell with its lumen end exhibiting signs of cytolysis. Kolatchev.

13. A portion of a cross-section of a salivary gland from a quiescent larva showing the lumen space filled with disintegrated cytoplasm; part of the epithelial cell or cells are still recognizable, in which are seen the nucleus, Golgi-material-secretion complexes, mitochondria, and storage granules. Mann–Kopsch–Altmann. M.T. = mitochondria.

14. A cross-section through a young salivary gland showing one of the cells releasing secretory material and part of its cytoplasm into the lumen which is partially filled with the discharge. Kolatchev.

15–16. Two young cells showing the condition of mitochondria in such cells. Benda–Baker.

During cytolysis, when the larva has become quiescent, or even a few hours before that, the cell and the material of disintegration lying in the lumen look somewhat as shown in Text-fig. II. 13. It shows a cell in which cytolysis has already touched the inner tip of its nucleus. The mass of disintegrated material from the glandular cells practically fills the whole lumen. The clefts in the mass of disintegrated cytoplasm are probably due to the technique to which the material had been subjected. In what is left of the cell Golgi bodies are rather numerous and do not show any sign of disintegration. Chondriomites are also numerous and they no longer aline themselves into threads so often as in younger cells. There are also granules, of course, and they stain in various shades of colour. Roughly, their ability to stain decreases as they become bigger. It is interesting to observe that once nuclear material, cytoplasm, and cytoplasmic inclusions are in the lumen of the gland, they soon lose not only their morphological identities but also their individual stainability which they possessed when still within the cell.

*Mitochondria.* Mitochondria appear in the form of both chondrioconts and chondriomites in the salivary gland-cells of all stages of development. They are numerous and are distributed evenly throughout the cell. In very young cells the chondrioconts of a granular construction seem to be more numerous than the other form. The chondriomites are not uniform in size. Some of them have a diameter of about the thickness of the chondrioconts, but there are others which are considerably bigger (Text-fig. II. 15 and 16). Vacuoles and droplets are also seen in mitochondria slides, although no evidence could be observed which would incline one to attribute a mitochondrial origin to them. In a more advanced stage the cells show more and bigger chondriomites. Some of the bigger ones appear to have a diameter four to five times the thickness of the filamentous form. The small chondriomites are also present and the two extremes are connected by a graded series (Text-fig. III. 17 and 18). Text-fig. III. 19 shows an interesting phenomenon which, up to this stage of development, is yet new. There appear among the bigger chondriomites some which do not stain so deeply as the rest. These lighter-coloured ones reach a rather big size as the cell advances towards full development, and it seems also there is a rough parallelism between the size and the ability to stain of these transforming chondriomites: power to stain lessens as their size increases. This situation is best brought out by viewing Text-fig. III. 17–22 in rapid succession. And Text-fig. IV. 23 and 24 represent two cells in which the enlarged and lighter-coloured chondriomites, if they can still be so called, grade imperceptibly into the fully formed granules which take only a faint trace of colour, if any at all. These are the granules which will eventually fill up the cell, giving the characteristic cytological appearance to the cells of glands of a fully grown larva regardless of whether the material has passed through fixatives suitable for mitochondria, for Golgi material, or for the nucleus and the respective subsequent treatments. Indeed, it is difficult for anybody who has seen such cells to attribute the origin of these granules to any organelle in the cell other than mitochondria.
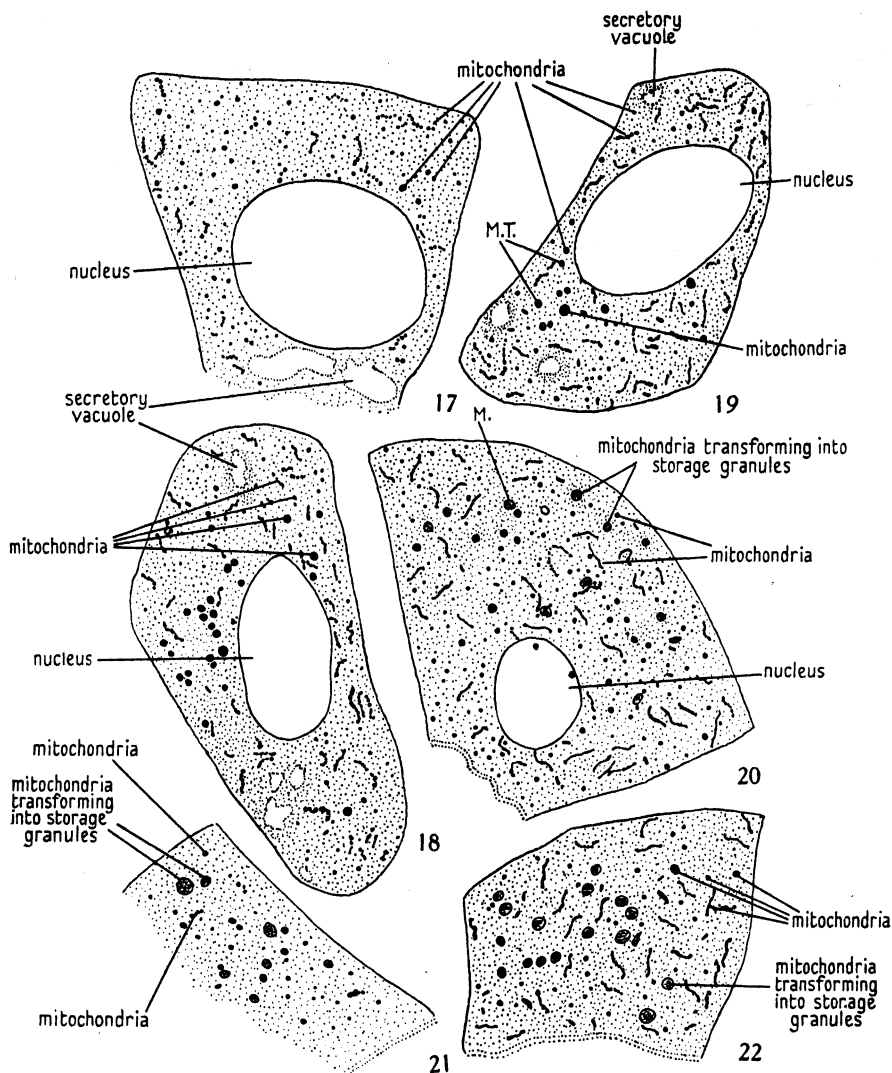
TEXT-FIG. III

17. A fairly young cell in which some of the chondriomites have attained a diameter four to five times that of the small ones. Note the two big vacuoles of secretion near the broken lumen end of the cell. Benda–Baker.

18. A cell in which some of the chondriomites have attained even greater size, though all of them still stain as intensely as the smaller ones. Benda–Baker.

19. A cell showing the enlarged chondriomites no longer taking mitochondrial stain as intensely as the smaller ones. Benda–Baker. M.T.=mitochondria.

20-2. Three cells in which the light-staining or transforming chondriomites have become more numerous. Benda–Baker. M=mitochondria transforming into granules.

Text-fig. IV. 25 shows a somewhat puzzling phenomenon. Rather frequently some granules may be encountered in a cell, usually in one filled, nearly or completely, with storage granules, which show splashes of intensely blue or faintly red colour according to the stain applied. Some may exhibit a coloured crescent capping partly around them. This phenomenon is observed only in cells one or two steps earlier than the stage of cytolysis. It must be regarded as one stage in the transformation from mitochondria to the mature granules, since, in cells having already begun to undergo cytolysis, blotched granules have never been seen (Text-fig. IV. 26). It is difficult to explain such granules unless one assumes that there are two methods for the chondriomites to transform themselves into granules. One is a uniform transformation throughout the granules; and the other an uneven one, leaving, before the process is completed, certain sections in the grain still of the chemical composition of mitochondria. Even so, however, it would still be difficult to explain the fact that the blotches on the granules often seem to stain more intensely than the tiniest chondriomites found in the same cell. Whatever the true nature of such blotches and crescents may be, the evidence pointing to a chondriomite origin for the granules is nearly irrefutable.

## DISCUSSION

In the salivary glands of the larva of *Chironomus*, neither Parat and Painlevé (1924), nor Beams and Goldsmith (1930), nor Gatenby (1932) saw any evidence that either Golgi bodies or mitochondria play a part in secretion synthesis. Beams and King (1932) came to the same conclusion in the salivary glands of the grasshoppers. Beams and Wu (1929), in their studies on the spinning glands of *Platyphylax designatus*, had the following to say: 'Throughout the activity of the gland, the Golgi apparatus changes little from normal condition, both in form and distribution. No relation of Golgi apparatus to the secretory phenomenon is apparent.' Wu (1930) reported that in the spinning glands of the larva of *Galleria mellonella* the secretory substance has a nuclear origin and that mitochondria play a negligible or at most a minor role. He could not detect any sign that Golgi bodies play a part in secretory synthesis. Lesperon (1937) also disclaimed any role by the two cytoplasmic elements in the synthesis of secretion in the silk glands of silk-worm and other insects. Baptist (1941) studied the salivary glands of an extensive list of Hemiptera-Heteroptera. He found in *Notonecta*, for instance, that the cells have developed the mechanism of forming zymogen-granules and are thus capable of producing a large quantity of secretion at short notice. To store this, the cells have also developed collecting vacuoles. But he also said that although both Golgi bodies and mitochondria typical of insect tissue are present in the cells, they 'bear no special relation to the nucleus or secretion granules'. He published, however, no figures of either Golgi material or mitochondria as seen in his material.

The result of my study on the salivary glands of *Drosophila* larvae, however, very clearly indicates that the secretory droplets are elaborated in the Golgi
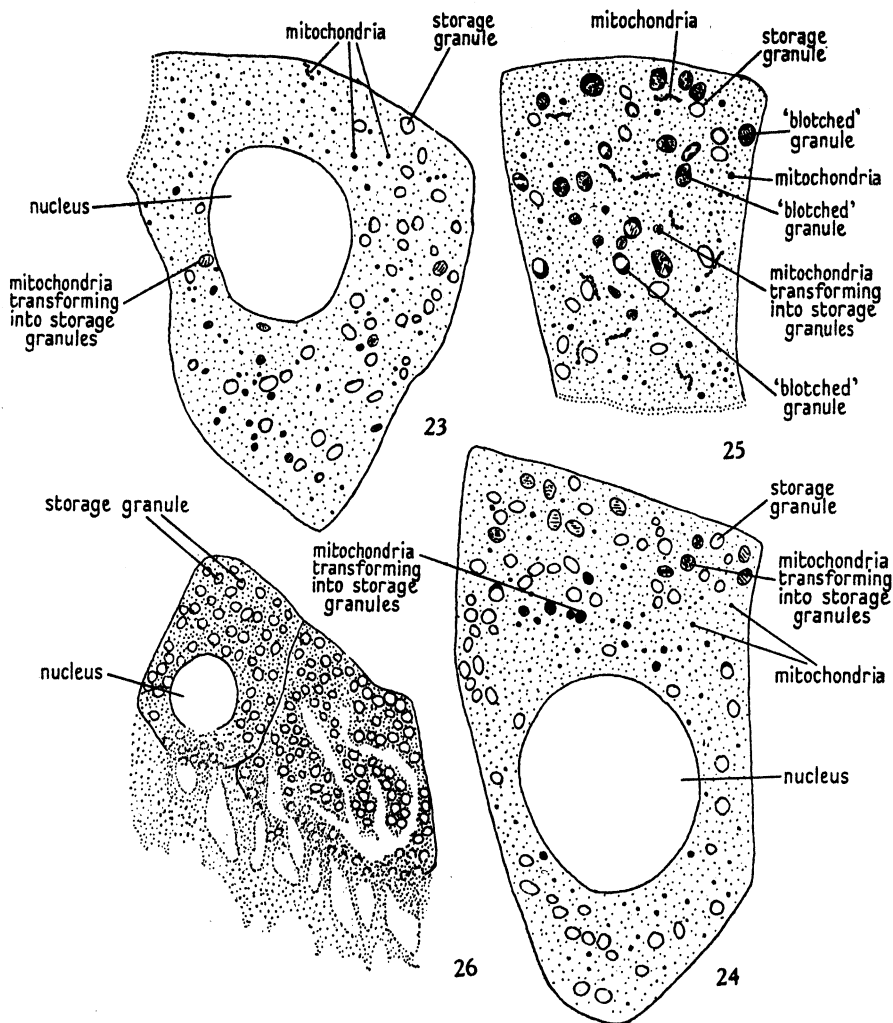
TEXT-FIG. IV

23-4. Two cells showing transition from late stages of transforming chondriomites to slightly coloured or colourless storage granules. In Fig. 24 all the stages of transition from intensely stained minute chondriomites to colourless storage granules are shown. Benda–Baker.

25. A cell containing 'blotched' storage granules. Benda–Baker.

26. Two cells undergoing cytolysis, their lumen ends broken and their disintegrated cytoplasm lying in the gland lumen. Only storage granules are shown in the cytoplasm. Kolatchev.

bodies. Briefly, the individual Golgi bodies are small roundish or irregularly shaped bits, and as each increases in size a lighter area (secretory droplet) appears in it, forming what I called in my previous study (1947) a Golgi-material-and-secretion complex. When a complex has reached a certain size the drop of secretion is released into the cytoplasm, and, as a result of the fusion of two or more of them, larger vacuoles are formed and stored in the cell. The Golgi-material shell now has broken into several small pieces, each of which presumably is potentially capable of elaborating another droplet of secretion. It may be said that so far as the Golgi material is concerned, its morphology and behaviour, in relation to the elaboration of secretion as observed in the cells of the salivary glands, almost exactly duplicate what has been seen in the cells of the glandular portion of the proventriculus (Hsu, 1947). I have seen no indication whatever that the secretory material is first separated under the influence of the mitochondria and then moved up to the Golgi material to be matured into secretory droplets. Neither have I seen anything which may suggest a nuclear or nucleolar origin for them. The only difference in the two cases is that in the proventriculus cells fusion of smaller droplets into bigger ones was not observed. This, however, is really a point of difference between the two types of cells in their methods of discharging their secretory material and does not concern the question of the origin of secretion.

With regard to the structure of Golgi bodies, I can only restate briefly what I have already said in my other paper (1947). My observations would only allow me to recognize the existence of a homogeneous substance, which I prefer to call Golgi material. This I believe corresponds to the 'Praesubstanz' of Hirsch (1932), the dense lipoid-containing substance of Baker (1944), and the osmiophilic or agentophilic substance of others. When a light area appears in a piece of Golgi material I have so far simply regarded it on observable evidences as a drop or granule of secretion viewed through a film of Golgi material, and have preferred to call the whole by the clumsy name 'Golgi-material-and-secretion complex'. It may be noted here that in my paper dealing with the midgut epithelium of *Drosophila* larvae (1947) I compared the structure of a 'complex' to a Golgi-material bowl in the hollow of which is set a drop or globule of secretion. But in spite of the lack of a visible film over the apparently exposed area of the secretion in some cases, my study since then has given me confidence to regard the structure of all the complexes at all stages as a vesicle. I have seen nothing which would make it necessary for me to differentiate a piece of Golgi material into a chromophilic and a chromophobic component at any stage of its activity.

In cells crowded full with granules the cytoplasm is squeezed into very narrow films whose widths are clearly indicated by the presence in them of chondriomites and chondrioconts. These elements are seen deposited around the granules; and in sections in which the granules are not sufficiently clearly coloured, it is by means of the distribution of the chondriome that the boundary of the individual granules is made out. It appears reasonable to say that this paucity of cytoplasm in the cell toward the end of the life of a

larva is at least one of the contributing factors why in cells packed full with granules hardly any droplets or vacuoles (indicating elaboration of digestive secretion) or broken lumen end (indicating discharging of secretion) could be observed.

This, however, is not the case in younger cells in which the granules either have not yet made their appearance or have not yet become so numerous and big as to occupy most of the space within the cell. In such cells there are observed more free droplets and vacuoles and more Golgi bodies containing secretion. Cells with broken inner wall and gland lumens filled with discharged secretion are much more often encountered in young glands or that section of older ones where comparatively younger cells are found. The larval life of *Drosophila*, except when moulting, may be said to be a period of ceaseless feeding. The cells of a salivary gland, up to a certain size, appear to go through a continual process of elaborating digestive fluid and storing it in vacuoles, ready to discharge it by means of a merocrine mechanism into the gland lumen whenever conditions demand. There does not seem to exist in them a clear-cut secretory cycle as has been observed both in the cells of the glandular portion of the proventriculus and the anterior portion of the midgut (Hsu, 1947).

As to the granules which give to the mature cells their characteristic and striking appearance in histological preparations, it appears that to secrete digestive enzymes is but one of the functions of the salivary glands in a *Drosophila* larva. When the cells have grown to a certain size another function, that of storage, begins to go on simultaneously with the function of secretion. Storage material in the form of granules begins to be transformed from individual chondriomites. I regard these granules as storage food, and not as having anything to do with digestion, on the basis of the following observations: (1) they have never been seen discharged into the gland lumen during the whole feeding period of the life of a larva; (2) they are seen to disintegrate into a homogeneous mass and to be set free into the lumen space only some time after the larva has ceased feeding; (3) unless the cell is filled with them, they are always seen accumulated at the basal end of the cell, while the secretion droplets and vacuoles have often been observed at the lumen end.

Regarding the storage granules found in older salivary gland cells, it is worth while to note that Painter (1945), in his interesting work on secretion with reference to the origin of cytoplasmic nucleic acid, apparently took these granules to be globules of digestive secretion. In describing his Fig. 17 he said it 'is filled with clear alveoli which are interpreted as secretory product which have not yet passed out of the cell'. But his Figs. 17 and 20 present to me the very familiar appearance of cells filled with what I now have evidence to regard as storage granules. His Fig. 21 is also convincingly that of a cell undergoing cytolysis, and does not, as he apparently then thought, represent a mere discharging of the 'secretory globules'. I have never seen these granules discharged into the lumen of the gland unless as a result of cytolysis

when the larva is about to pupate. The larva gland cells do not 'secrete' these granules and then empty them into the gland lumen and begin to elaborate another batch and so on. The formation and accumulation of these granules proceed as a linear process and are not carried on in the cell in cycles. They visibly begin in the fairly young cells and stop presumably after the larva has ceased feeding. I have seen histolyzing glands from larvae which have stopped feeding but are still very actively crawling about before becoming quiescent. It would seem that the stored food is drawn upon almost immediately after food intake is stopped. Salivary glands are apparently the first organs to cease functioning in metamorphosis, since as organs they no longer answer any need of the organism at the time. It would be interesting, therefore, to re-examine the nucleic acid situation, correlating the amount and distribution of it not with the larger granules found in the older cells but with the smaller droplets and vacuoles found in the younger cells.

Ross (1939), in her developmental study of the salivary glands in the larva of *Drosophila melanogaster*, also misinterpreted the large granules to be globules of digestive secretion, though she reported that the salivary gland cells of adult *Drosophila* show no such 'globules'.

SUMMARY

1. The salivary glands in the larvae of *Drosophila* show evidence of serving two functions: (1) production of digestive secretion, (2) accumulation of reserve food for the period of pupation. The two functions proceed simultaneously within the same cell during certain stages of its development.

2. A single droplet of digestive material has been seen to originate and grow within each Golgi body in the gland-cells. When a certain size is reached the droplet is released into the cytoplasm and by the fusion of two or more of them bigger vacuoles are formed. The secretory material is discharged into the lumen by means of a merocrine mechanism. Neither mitochondria nor nucleus has been observed to take any visible part in the elaboration of secretion droplets.

3. The storage granules found in older and larger cells have been observed to be direct transformations of chondriomites, and neither the Golgi material nor the nucleus shows any sign of participation in the formation of these granules.

4. From the standpoint of morphology and behaviour, the Golgi bodies found in the salivary gland cells are the same as found in the cells of the glandular portion of the proventriculus and the epithelium of the anterior portion of the midgut of the larva.

5. My observations do not lend themselves convincingly to a two-component conception of the structure of Golgi bodies.

# REFERENCES

BAKER, J. R., 1944. Quart. J. micr. Sci., **85,** 1.

BAPTIST, B. A., 1941. Ibid., **83,** 91.

BEAMS, H. W., and GOLDSMITH, J. B., 1930. J. Morph., **50,** 497.

—— and KING, R. L., 1932. Ibid., **53,** 59.

—— and WU, C. F., 1929. Ibid., **47,** 261.

BODENSTEIN, D., 1943. Biol. Bull., **84,** 13.

GATENBY, J. B., 1932. Amer. J. Anat., **51,** 253.

HIRSCH, G. C., 1932. Z. Zellforsch. mikr. Anat., **14,** 517.

HSU, W. S., 1947. J. Morph., **80,** 161.

LESPERON, L., 1937. Arch. Zool. exp. gén., **79,** 1.

MAKINO, S., 1938. Cytologia, **9,** 272.

PAINTER, T. S., 1945. J. exp. Zool., **100,** 537.

PARAT, M., and PAINLEVÉ, J., 1924. C.R. Acad. Sci., **179,** 612.

—— 1924. Ibid., 543.

ROSS, F. B., 1939. J. Morph., **65,** 471.

SONNENBLICK, B., 1940. Genetics, **25,** 137.

WU, C. F., 1930. J. Morph., **49,** 509.

# Phosphatase Activity of *Drosophila* Salivary Glands

BY

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THE presence of alkaline phosphatase in chromosomes has been demonstrated by means of histochemical staining methods (Danielli, 1946; Krugelis, 1942, 1946). These studies indicated the effects of various fixatives and substrates on the subsequent staining reactions. The present study presents certain quantitative aspects of the effects of acetone, alcohol, and pH on the phosphatase activity of whole salivary glands of *Drosophila*.

## MATERIAL AND METHODS

Paranitrophenyl phosphate (Bessey, Lowry, and Brock, 1946) was used as a substrate. Magnesium was omitted after failure to detect increased activity with added magnesium. Hydrolysis was proportional to enzyme concentration under the conditions employed. In order to reduce the rate of inactivation of the enzyme the determinations of alkaline phosphatase activity were made at pH 8·6 rather than at the more alkaline optimum of maximal activity.

To determine the general characteristics of the phosphatases present in this material, several dozen glands were pooled and attempts made to get reproducible extracts and suspensions. This method was unsatisfactory. The salivary glands vary greatly in size and enzyme activity from one individual to another; but the single glands of a pair appear to be identical in size and enzyme content. Accordingly it is possible to control factors such as differential extraction of the enzymes and individual variation as follows: provided that enzyme blanks (the paranitrophenol colour value of samples without substrate) are negligible, as is the case here, each larva provides two aliquots. A pH curve or other comparison can then be run as indicated in Table I, where letters represent larvae and postscripts the glands of the pair.

### TABLE I

| pH | 4·6 | 5·0 | 5·4 | 5·8 | &c. |
|---|---|---|---|---|---|
| Samples   .        .        .        . | A1 | A2 B1 | B2 C1 | C2 D1 | &c. |
| | K1 | L1 | K2 | L2 | |

Individual glands were dissected in saline and fixed in acetone in tubes $6 \times 25$ mm. The acetone was replaced three times during the first 10 minutes; at the end of the fixation period all but 1·0 c.mm. of acetone was removed and 50 c.mm. of buffer added (M/25 diaminobutane pH 7·3–10·2, or barbital, pH 3·0–7·3). After a suitable extraction period 50 c.mm. of substrate (adjusted to the pH of the extraction buffer) were added. The preparations were incubated 1–3 hours at 30° C., depending on size of gland. The gland was removed with a glass needle and the reaction stopped by addition of 15 c.mm. of 1·0 N. NaOH. The optical density of 100 c.mm. samples was measured on a Beckman spectrophotometer using Lowry micro-cells. Enzyme blanks were run on some pairs in each series but their colour was invariably negligible. The glands remained intact through several consecutive determinations.

## OBSERVATIONS

*Fresh extracts.* When fresh glands were placed in distilled water or buffer for varying periods prior to addition of the substrate very little enzyme activity was found. In one case 20 glands were placed in 100 c.mm. of distilled water and subjected to the gentle pounding of an electromagnetic stirring bead for 2 hours. In this instance less' than 10 per cent. of the enzymatic activity of the acetone-fixed aliquot was found. In preliminary experiments it was found that whenever, during dissection, a cell of a gland was penetrated by the dissecting needle, the subsequently determined activity of the gland was higher than that of the uninjured gland (cf. Table IV, B3 and B4). It is evident that either the cell membrane or more probably the membranous capsule of the gland effectively prevents extraction of the enzyme and entrance of the substrate even in unphysiological media.

This fairly obvious property of membranes has occasionally been measured (Linderstrøm-Lang and Holter, 1933, and Doyle, 1938). When the gland is frozen (Table II, B1, B2; C1, C2) or is fixed (Table II, Q1, Q2; R1, R2) this property of the gland surface is diminished.

TABLE II. *Phosphatase Activities of Individual Salivary Glands*

| Gland | Treatment | pH | Activity |
|---|---|---|---|
| B1 | Fresh | 8·6 | 0·90 |
| B2 | Frozen | 8·6 | 1·37 |
| C1 | Fresh | 8·6 | 0·160 |
| C2 | Frozen | 8·6 | 0·400 |
| Q1 | Fresh | 8·6 | 0·047 |
| Q2 | Acetone 20 min. | 8·6 | 0·321 |
| R1 | Fresh | 8·6 | 0·056 |
| R2 | Acetone 24 hrs. | 8·6 | 0·400 |
| S1 | Acetone 24 hrs. | 9·5 | 0·700 |
| S2 | Acetone 24 hrs. | 8·6 | 0·472 |
| Fat | Acetone 24 hrs. | 8·6 | 0·035 |

Fat is found closely applied to the glands and it is sometimes difficult to remove all of the fat without injuring the glands. That small traces of fat contain negligible quantities of phosphatase is shown in Table II, where pieces of fat twice the volume of the glands of specimen S were assayed along with the glands.

*Fixed glands.* Several pairs of glands were fixed in acetone for 24 hours and allowed to stand in distilled water for 15 minutes. Negligible activity was found in the extract and the activity of the extracted gland corresponded with the activity in glands not extracted. When extracted for 24 hours at 5° C. in M/25 diaminobutane buffer pH 9·5, about one-third of the total activity was found in the extract. In another series glands fixed in acetone for 9 days and extracted for 16 hours at 5° C. with M/50 barbital pH 7·3 gave 20 per cent. of the total activity in the extract. In this series redetermination of the residual phosphatase activity gave 87 per cent. of the initial residual activity. The alternate glands of pairs treated at the same pH with diaminobutane buffer gave corresponding results.

The total enzymatic activity of glands fixed in acetone is higher (at pH 4·4 and 8·6) than that found after similar treatment with 80 per cent. alcohol (Table III). Here 75 per cent. of the pH 8·6 acetone value was found after

TABLE III. *Phosphatase Activities of Glands after Fixation in 80 per cent. Alcohol and in Acetone at pH 8·6*

| Gland | Time of fixation | | Observed activity | pH | Notes |
|-------|---------|---------|---------|------|-------|
|       | Alcohol | Acetone |         |      |       |
| H1    | 2 hrs.  | . .     | 0·408   | 8·6  | = 70 per cent. of H2 (Alk-p-ase). |
| H2    | . .     | 2 hrs.  | 0·590   | 8·6  |       |
| J1    | 1½ ,,   | . .     | 0·175   | 8·6  | = 75 per cent. of J2. |
| J2    | . .     | 1½ ,,   | 0·235   | 8·6  |       |
| K1    | 2½ ,,   | 22 ,,   | 0·185   | 8·6  | = 77 per cent. of K2. |
| K2    | 0       | 24 ,,   | 0·248   | 8·6  |       |
| L3    | 2½ ,,   | 22 ,,   | 0·119   | 8·6  | = 73 per cent. of L4. |
| L4    | 0       | 24 ,,   | 0·171   | 8·6  |       |
| M7    | 2½ ,,   | 22 ,,   | 0·110   | 4·4  | = 20 per cent. of M8 (Acid p-ase). |
| M8    | 0       | 24 ,,   | 0·585   | 4·4  |       |

80 per cent. alcohol treatment and 20 per cent. of the pH 4·4 acetone value. Since the results obtained with specimens J and H might be ascribed to a differential effect of acetone and alcohol on the penetrability of the cellular material, the subsequent specimens (K, L, M) were treated with acetone as well after the alcohol fixation.

*Redeterminations on the same gland.* Using whole acetone-fixed glands, only a fraction of the enzyme is extracted during the initial determination. A second determination may then be made under similar or altered conditions to examine the effect of the first determination on the residual enzyme.

Representative data from these experiments are given in Table IV. Here it would appear that 60 per cent. of the pH 8·6 activity survives a determination (Detn.) at pH 4·4; that 45 per cent. of the pH 4·4 activity survives a determination at pH 8·6; that 38 per cent. of the pH 8·6 activity survives a determination of pH 8·6; and that 87 per cent. of the pH 7·3 activity survives a determination at pH 7·3. The decreased activity after the more alkaline initial determinations is in keeping with other findings (Lundsteen and Vermehren, 1936) in which the pH optimum is more alkaline for short digestion times than for longer ones.

TABLE IV. *Redetermination of Phosphatase Activities on Glands fixed in Acetone for 24 Hours*

| Gland | pH | | Activity | | Notes |
|---|---|---|---|---|---|
| | Detn. 1 | Detn. 2 | Detn. 1 | Detn. 2 | |
| T1 | 7·3 | 7·3 | 0·990 | 0·870 | $\frac{870}{990}$ = 87 per cent. of p-ase after neutral Detn. |
| A1 | 4·4 | 8·6 | 0·317 | 0·244 | $\frac{244}{413}$ = 60 per cent. of Alk-p-ase after acid Detn. |
| A2 | 8·6 | 4·4 | 0·413 | 0·150 | $\frac{150}{317}$ = 45 per cent. of Ac-p-ase after alk. Detn. |
| B3 | 8·6 | 8·6 | 0·351 | 0·133 | B4 punctured with needle. $\frac{133}{351}$ and $\frac{183}{462}$. |
| B4 | 8·6 | 8·6 | 0·462 | 0·183 | = 38–9 per cent. of Alk-p-ase after alk. Detn. |

Gomori preparations for both acid and alkaline phosphatase made subsequently to the initial quantitative determinations showed little differences from those made immediately following the respective fixations.

## DISCUSSION

That phosphoesterases from different sources vary with respect to stability to acetone and to alcohol is indicated by comparison of these results with those of Stafford and Atkinson (1948, rat tissues), who found higher alkaline phosphatase activity after alcohol (80 per cent.) than after acetone fixation.

The absence of appropriate values for fresh extracts and homogenates in this study prevents an estimation of the degree of destruction of alkaline and acid phosphatase by the alcohol and acetone. Determination of the ratio of activities found to true enzyme content requires the preparation of suitably cytolysed samples without major losses.

It is obvious, however, that acetone preserves more phosphatase activity in our material than does alcohol.

In specimen B, Table IV, B4 was deliberately punctured during dissection with resulting higher activity. The amount of enzyme lost between deter-

mination 1 and 2 was essentially the same in glands B4 and B3 (unpunctured). This would indicate that the increased activity of B4 over B3 is due to better access of substrate, and that at pH 8·6 (for 2 hours) the decreased activity in determination 2 as compared to the initial determination is due to inactivation rather than to extraction of the enzyme.

With sectioned rabbit material (Doyle—in the press) there is much greater extraction of enzyme (especially of acid phosphatase) than from these whole glands. Furthermore, acid phosphatases from many sources are much more labile than alkaline phosphatases. Gomori (1946) used acetyl cellulose to reduce losses and translocation of enzyme. A coating on the section which prevents diffusion of the enzyme will to some degree also affect the rate of penetration of the substrate. The problem then arises as to whether, in the absence of completely cytolysed cells in extracts and in the absence of free access of substrate to cell structure in sections, the activities observed under given experimental conditions can represent the enzymatic contents of the material. Only when the true enzymatic contents of cellular structures can be ascertained will there be a better basis for conjecture concerning the role of the enzymes, but the demonstration of maximal enzymatic *content* will not of itself indicate the normal physiological *activity* with respect to that enzyme.

### SUMMARY AND CONCLUSIONS

The phosphatases in the cytoplasm and nuclei of *Drosophila* salivary glands are better preserved by fixation in absolute acetone than in 85 per cent. alcohol. In whole glands there is relatively little extraction of the enzyme during assay. Phosphatase activity is more resistant to incubation at neutrality than at pH 8·6, but in this material there is sufficient residual enzymatic activity to permit redetermination of alkaline, neutral, or acid phosphatase activity by staining methods after an initial quantitative determination. The state of the membranes of the gland affects the penetration of the substrate sufficiently to limit the activities obtained.

### REFERENCES

BESSEY, O., LOWRY, O., and BROCK, M., 1946. J. biol. Chem., 164, 321.
DANIELLI, J. F., 1946. (Brit.) J. exp. Biol., 22, 110.
DOYLE, W. L., 1938. J. cell. comp. Physiol., 11, 291.
—— 1948. In the press.
GOMORI, G., 1946. Amer. J. clin. Path., 16, 347.
KRUGELIS, E. J., 1942. J. cell. comp. Physiol., 19, 377.
—— 1946. Biol. Bull. (Woods Hole), 90, 220.
LINDERSTRØM-LANG, K., and HOLTER, H., 1933. Compt. rend. Lab. Carlsberg. ser. chim., 19, no. 13, 1.
LUNDSTEEN, E., and VERMEHREN, E., 1936. Ibid., 21, 147.
STAFFORD, R. O., and ATKINSON, W. B., 1948. Science, 107, 279.

# The Accumulation of Carotenoids in the Golgi Apparatus of Neurones of *Helix, Planorbis,* and *Limnaea*

BY

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## INTRODUCTION

THE existence of coloured granules in neurones of pulmonate gastropods is well known through the work of Legendre (1909) and others. The coloured matter has been usually described as 'lipochrome', a term that Lison (1936) regards as bad since it covers two distinct chemical classes (the carotenoids and the chromolipoids) and has been used in different ways by different authors.

Thomas (1948) has recently produced evidence for considering these coloured granules as a Golgi product, a view not in agreement with that regarding the Golgi apparatus advanced by many workers.

This paper presents the results of a histochemical examination of the coloured substance, and a repetition of some of Thomas's work. The coloured granules contain carotenoid and appear to be formed in the interna of the Golgi bodies.

## MATERIAL AND METHODS

Thomas worked with the cerebral ganglia of *Helix aspersa*, in which many of the neurones are very large. The amount of coloured matter is usually small even in the largest cells; there is very much more in the neurones of *Limnaea stagnalis* (L.) and *Planorbis corneus* (L.), both very common freshwater snails. In the former the central nervous system is distinctly coloured by the pigment. In the latter the bright red coloration is due to granules inside the cells and to a certain amount of haemoglobin in the blood as well.

The following histochemical methods were employed:

(i) For lipoids in general, material was fixed in formal-calcium, and frozen sections were cut and coloured with sudan black B (Baker, 1944). Sudan black is specific for lipoids (Lison, 1936; see also Cain, 1947*a*). It does not colour pure carotenoids (Lison, 1936, p. 245) and, of course, it does not colour *solid* lipoids. Lison includes the carotenoids under pigments. As they are soluble in lipoid-solvents, they are included here under the heading of lipoids.

(ii) Baker's acid haematein test for phospholipines (Baker, 1946, 1947; Cain, 1947*b*) was used, with pyridine extraction as control.

(iii) For detection of carotenoids the Carr–Price reaction (Carr and Price, 1926) was used. With antimony trichloride ($SbCl_3$) in chloroform carotenoids and vitamin A give a blue coloration which is not permanent.

In addition, the granules were tested with concentrated sulphuric acid, and with iodine (Lison, 1936, p. 245). With these reagents carotenoids give a deep-blue colour.

As a supplementary test, sections were exposed to light and air and the rate of fading was noted.
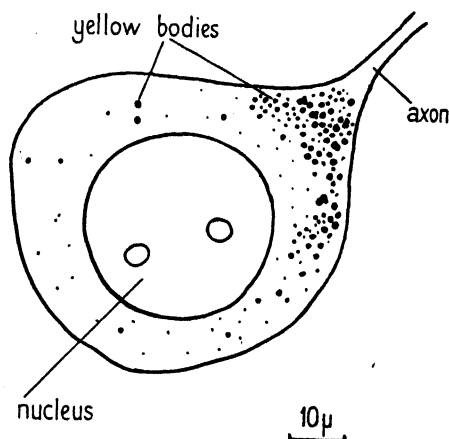


Text-fig. 1. Diagram of the distribution of bodies visible in a neurone of *Helix*.

Living cells were observed in sodium-calcium saline (Baker, 1944), and were stained supravitally with neutral red chloride, methylene blue (BDH), nile blue, and Janus green B (Höchst).

Mann–Kopsch preparations were made of neurones from all three species, and Thomas's variant of the Mann–Kopsch technique was used with *Planorbis*.

*Helix* neurones were fixed in Helly, postchromed, stained with Altmann's acid fuchsine, differentiated with sodium carbonate solution, and counterstained with methyl blue (Cain, 1948).

### RESULTS

In living neurones of *Helix* a large number of bodies can be seen, which are either subspherical or irregular. These latter are described by Thomas as mulberry forms. The largest are often distinctly yellow in colour. These bodies are scattered throughout the cell, but more and more thickly towards the axon hillock, in which there is usually a large number (Text-fig. 1). On close examination there may be seen a cap or a granule or several granules

adhering to the rim of such bodies. In *Limnaea* and *Planorbis* these bodies are more evident, very numerous, and quite brightly coloured yellow, the largest being a faintly reddish or brownish yellow, and rather more irregular than in *Helix*. Their distribution is the same.

With methylene blue (1 in 10,000) both the bodies and their associated granules or caps are stained, the latter very deeply. A similar effect is produced, but less clearly, with neutral red chloride and with nile blue. Neutral red is the least satisfactory with *Planorbis* and *Limnaea* because in them
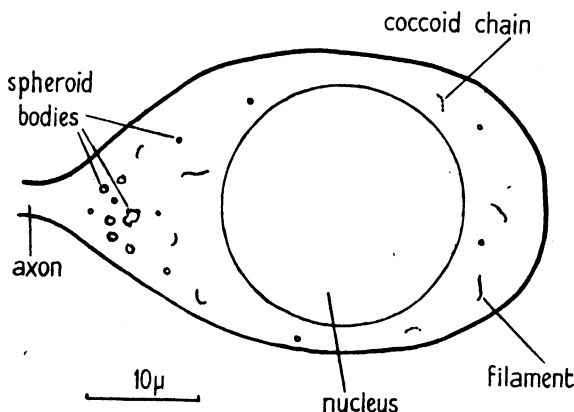


Text-fig. 2. Diagram of the relation between the filaments and coccoid chains shown with Janus green, and the spheroid bodies.

nearly all the bodies are already coloured and the contrast is not great. Methylene blue is very satisfactory.

When neurones were exposed to the vapour of osmium tetroxide, the rims of these bodies, together with their caps and granules, become blackened. Prolonged action results in the blackening of the whole body.

Minute filaments and coccoid chains were not distinctly seen in living cells. On application of Janus green B (Höchst), after many attempts, very distinct filaments were seen in living cells of *Helix* (*Planorbis* and *Limnaea* neurones were not investigated because of the multiplicity of spheroids which make for obscurity). These were very thin and short, sometimes bent or kinked. Coccoid chains were seen most distinctly in a damaged cell; it is possible that they are filaments beginning to break up. In distribution the filaments do not resemble the spheroid bodies, which are also clearly visible, and are not associated with them. They are scattered throughout the cytoplasm and show no tendency to concentration in the axon hillock (Text-fig. 2). They were seen in only a few cells, lying next to those on the outside of the teased-out cell-mass, which were dead and stained diffusely.

Mann–Kopsch preparations were made of neurones of all 3 species, 6 days being found a suitable time of osmication. In preparations from *Helix*.

batonnettes as usually described were to be seen in nearly all cells, sometimes with an associated 'archoplasm' but quite often without. Sometimes the cytoplasm contained nothing else, but on occasion there were black or grey spheroidal bodies. These might occur anywhere in the cytoplasm, but where cells were cut through the nucleus and axon hillock it could sometimes be seen that there was an aggregation in the hillock (Text-fig. 3). In preparations of *Limnaea* and *Planorbis* neurones much the same pictures were obtained, but in general the batonnettes were less obvious and the globules more so.
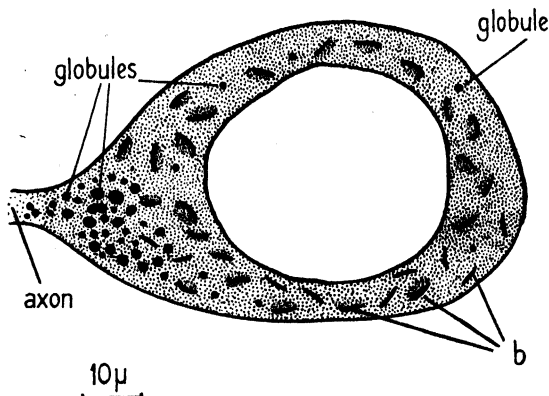


TEXT-FIG. 3. Diagram of the distribution of globules and batonnettes in a Mann–Kopsch preparation. *b*, batonnettes, mostly with archoplasm. Nucleus blank.

In distribution, the globules corresponded exactly to the coloured granules and complexes seen in the living cells. The batonnettes did not correspond to these and, as in *Helix*, were scattered throughout the cytoplasm.

Thomas describes only batonnettes in his Mann–Kopsch preparations, but was able to show globules by bleaching with Veratti's acid permanganate (followed by oxalic acid to remove the brown deposit of oxide) and then colouring with sudan black, when globules appeared in positions quite unrelated to those of the batonnettes which had been shown by osmium. When neurones of *Limnaea* and *Planorbis* were coloured with sudan black without previous bleaching, it could be seen plainly that the globules were much darker but the batonnettes were not; and when cells were bleached entirely and then coloured with sudan black, the globules reappeared in exactly their former sizes and positions but the batonnettes did not. It appears, therefore, that these globules, stained by osmium tetroxide, are the same as those produced by Thomas using sudan black after bleaching. Osmium-impregnation appears to be capricious. Either the batonnettes alone, or both batonnettes and globules may be stained. Thomas's variant (p. 455) of the Mann–Kopsch technique was tried on *Planorbis* neurones. Only globules were seen, except for a very faint indication of batonnettes in a very few cells. These globules agreed in all respects with those seen by the Mann–Kopsch technique.

With *Helix* material fixed in Helly's fluid, postchromed, stained with acid fuchsine as for mitochondria, and differentiated with sodium carbonate solution (Cain, 1948), no mitochondria were seen, but spheroid complexes with very fuchsinophil rims and colourless interna were present and agreed entirely with Thomas's description (p. 456).

With Baker's acid haematein test the picture obtained varied. The pyridine-extraction control always gave a completely negative result. The acid haematein test showed the cytoplasm either a clear yellow-brown with
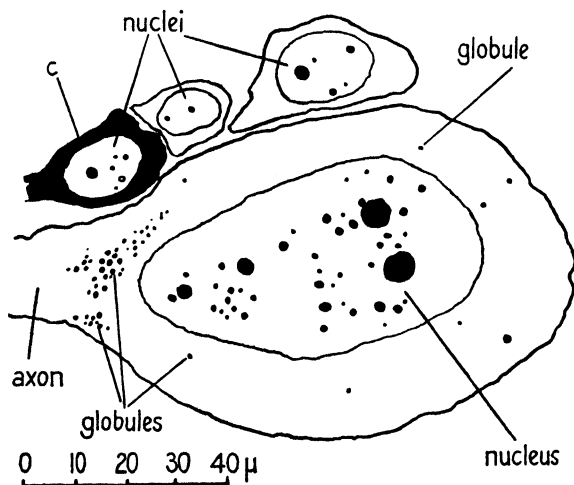


TEXT-FIG. 4. Camera lucida drawing of an acid-haematein preparation of *Helix* neurones. *c*, cell with the cytoplasm staining heavily throughout.

certain blue-stained bodies or intense blue-black throughout. In the latter case the cells were often slightly shrunken, and on occasion were confined to one part of the ganglion, within which every cell was blue-black. It is considered that such cells were damaged in some way and a lipophanerosis had taken place so that phospholipines were liberated in the cytoplasm. In the cortex of rat adrenals very similar appearances have been seen in those cells of the zona reticularis lying next to the medulla; this is a region in which necrotic cells are found. In this case, the liberation of phospholipine is a sign of the death of the cell.

In neurones not blue-black throughout, coloured spheroids are occasionally visible, again tending to be concentrated in the axon hillock (Text-fig. 4). In addition there are small coloured bodies, scattered in the cytoplasm, which appear to be the caps or rims associated with the smallest spheroid complexes in the living cell, the remainder of each complex being invisible in acid-haematein preparations. Some of the larger spheroids appear to have less coloured interna, but, as Thomas remarks (p. 452), this is not obvious, and

cannot be asserted with great conviction. Clouds of phospholipine occurred in some cells.

Material fixed for 3 days in formal-calcium was cut on the freezing microtome and the sections coloured with sudan black B. This method (Baker, 1944) showed large numbers of globules in the axon hillock, and others scattered throughout the cell. In every case it was the rim or attached granule that coloured with the sudan black. The picture was almost exactly that of living cells exposed to osmium tetroxide vapour. In all 3 species the cytoplasm coloured heavily throughout, and it was necessary first to remove this general colouring which indicates the presence of lipoids throughout the cytoplasm.

The yellow or orange-yellow pigment in the cells rapidly becomes colourless under the influence of light and air; the application of concentrated sulphuric acid produces a fine blue-green colour immediately. This had been noted by Smallwood and Rogers (1908), who concluded that the pigment was 'lipochrome'. Iodine in potassium iodide solution gives a deep violet. These facts indicate that the pigment is carotenoid, and this is confirmed by the blue colour given with a solution of antimony trichloride in chloroform. This reagent gives a blue colour with both vitamin A and carotene, and in spite of statements to the contrary cannot be used to distinguish between them unless heat is used (Andersen and Levine, 1935). As the chloroform tends to dissolve out the carotenoid, and heating accelerates this, it was found impossible to distinguish any colour on heating. The white precipitate of oxychlorides formed by antimony trichloride in contact with water tends to obscure the preparation.

In cells fixed for 6 hours in formal-calcium the carotenoid-containing granules take up far less sudan black than would fat-droplets of the same size. Lison (1936) notes that pure carotenoids are negative to lipoid reagents. This suggests that the largest granules are composed of carotenoids only among the lipoids, although the presence of proteins cannot be excluded. The granules in *Planorbis* and *Limnaea* often seem much more angular than those in *Helix* and give an impression of solidity.

It appears then that the complexes contain phospholipine and perhaps other lipoids in the rims, caps, or associated granules, and carotenoid in the interna. Vitamin A being much paler in colour than carotene, and giving the same reactions, cannot be excluded by the results given above, nor is its presence established. As there is no evidence for the manufacture of carotenoid by the Golgi apparatus as against its collection from other regions, it seems best to refer to its *accumulation* therein.

## DISCUSSION

The results given above agree with those of Thomas. It seems reasonable to conclude, as he does, that the filaments stained with Janus green appear in Mann–Kopsch preparations as the batonnettes and are the mitochondria. The spheroid-complexes are the Golgi bodies with their products. There is

a coincidence of structure and distribution-patterns of the globules (spheroid-complexes) seen in life and after treatment with all the following: neutral red, nile blue, methylene blue, acid haematein, Helly and acid fuchsine, formal-calcium and sudan black, and the Mann–Kopsch technique and Thomas's variant of it. This demonstrates clearly that they are all the same bodies. From the Mann–Kopsch preparations and the living cells stained with Janus green B it is clear that they are not associated with the mitochondria. Their identification with the Golgi apparatus is discussed by Thomas (p. 456). In structure they agree very well with bodies in other cells which are undoubtedly the Golgi apparatus (Worley, 1943, 1944, and 1946; Worley and Worley, 1943; Baker, 1944; Cain, 1947a).

The principal arguments that might be brought against this identification are that such bodies are not shown by the standard Golgi methods, that the batonnettes with their archoplasm are the Golgi apparatus, or that the use of neutral red chloride and other supravital stains cause the production of artifacts. That in these neurones the spheroid complexes are not shown by the standard methods is a much more serious objection. The Mann–Kopsch method does show it sometimes, less readily in *Helix* than in *Limnaea* and *Planorbis*, but it is quite easy to find cells in which nothing but the batonnettes can be seen. But the standard methods are wholly empirical, and, as far as is known, no Golgi apparatus is under obligation to appear when they are used. So very little is known about conditions at the inner surfaces of the cell and the conditions under which silver and osmium precipitates form. What is known about monomolecular layers on water-surfaces indicates that their behaviour can change very greatly with small changes in pH and the concentration of ions in the water (see e.g. Langmuir, 1934) and it would not be at all surprising if structures containing layers of oriented molecules should be very similar in morphology but very diverse in their behaviour, differing, particularly, in different sorts of cell, and most divergent in the most specialized. The fact that these bodies do not usually appear with the standard Golgi methods does not rule out the possibility that they are the Golgi apparatus. The objection to the use of neutral red and similar stains can hardly be upheld in this case because it is not a question of making structures visible, but of staining structures already visible in the cell.

The view that the batonnettes are the Golgi apparatus has been very well supported. This was based on their regular appearance with the standard Golgi methods, and a supposed homology with the lepidosomes of the pulmonate primary spermatocyte. But there seems to be no special reason for carefully selecting cells which show batonnettes only and excluding one showing the spheroids as well; and the batonnettes do correspond with the mitochondria as shown by Janus green, and do not correspond with the spheroid complexes seen in the living cell and ignored by upholders of the batonnette theory. In *Limnaea* and *Planorbis* the batonnettes are less readily shown by the Mann–Kopsch method than in *Helix*, and the spheroids perhaps more readily. Perhaps if investigations on gastropod neurones had started

with *Planorbis* instead of *Helix*, as much attention might have been given to the spheroids as to the batonnettes.

## SUMMARY

Repetition of some of Thomas's (1948) work on *Helix* neurones and its extension to neurones of *Planorbis* and *Limnaea* confirms his conclusions that the batonnettes shown by standard Golgi methods are mitochondria, and the Golgi apparatus is represented by spheroid complexes, scattered throughout the cell but tending to be concentrated in the axon hillock.

The spheroid complexes appear to consist of an externum, continuous or not, which contains phospholipine and possibly other lipoids, and an internum in which carotenoids are accumulated. This accumulation is greater in *Limnaea* and *Planorbis* than in *Helix*.

## REFERENCES

ANDERSEN, A. C., and LEVINE, V. E., 1935. Proc. Soc. exp. Biol. Med., **32**, 737.
BAKER, J. R., 1944. Quart. J. micr. Sci., **85**, 1.
—— 1946. Ibid., **87**, 441.
—— 1947. Ibid., **88**, 463.
CAIN, A. J., 1947*a*. Ibid., **88**, 151.
—— 1947*b*. Ibid., **88**, 467.
—— 1948. Ibid. (In the press.)
CARR, F. H., and PRICE, E. A., 1926. Biochem. J., **20**, 497.
LANGMUIR, I., 1934. J. Frank. Inst., **218**, 171.
LEGENDRE, R., 1909. Arch. Anat. micr., **10**, 287.
LISON, L., 1936. *Histochimie animale, Méthodes et Problèmes.* Paris (Gauthier-Villars).
SMALLWOOD, W. M., and ROGERS, C. G., 1908. J. comp. Neur. Psychol., **18**, 45.
THOMAS, O. L., 1947. Quart. J. micr. Sci., **88**, 269.
—— 1948. Ibid., **88**, 445.
WORLEY, L. G., 1943*a*. Proc. Nat. Acad. Sci., **29**, 225.
—— 1943*b*. Ibid., **29**, 228.
—— 1944. J. Morph., **75**, 77.
—— 1946. Ann. New York Acad. Sci., **47**, 1.
—— and WORLEY, E. K., 1943. J. Morph., **73**, 365.

# A Further Note on Nile Blue

BY

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IN a previous paper (Cain, 1947) it was shown that nile blue can be used to distinguish between acidic and non-acidic lipoids, and that lecithin stained very deep blue with a 1 per cent. solution, while oleic acid was only faintly coloured. Since lecithin stains, it was suggested that other lipines might stain blue also and that the oleic acid used might be impure.

A sample of phosphorus-free oleic acid was dried over anhydrous sodium sulphate for three months. One portion was then shaken with 1 per cent. nile blue aqueous solution, and solid nile blue was added to another. The acid in contact with the solution coloured blue immediately. The other portion showed only traces of blue (appearing red by transmitted light because of fluorescence) after 2 hours. After 12 hours it had darkened considerably, but did not reach equality with the first portion for 24 hours. Since the dried acid takes up nile blue so much more slowly than that in contact with water, it appears that the blue-staining of oleic acid is connected with its power of imbibing water, and as it is highly unlikely that dry oleic acid will be met with in animal tissues, no use can be made of blue-staining with 1 per cent. nile blue to differentiate between lipines and fatty acids.

A sample of pure galactolipine (phrenosin and kerasin) which gave a negative reaction with the acid haematein test was found to stain deep blue with 1 per cent. nile blue. The substance was attached to a coverslip by gentle warming and then cooling. Kaufmann and Lehmann (1926) obtained a negative result with pure phrenosin and with pure kerasin, probably because, as with lecithin, their method of impregnating pith was not suitable. Nile blue, therefore, cannot be used to distinguish between various members of the lipines.

## REFERENCES

CAIN, A. J., 1947. Quart. J. micr. Sci., **88**, 383.
KAUFMANN, C., and LEHMANN, E., 1926. Virchows Arch., **261**, 623.
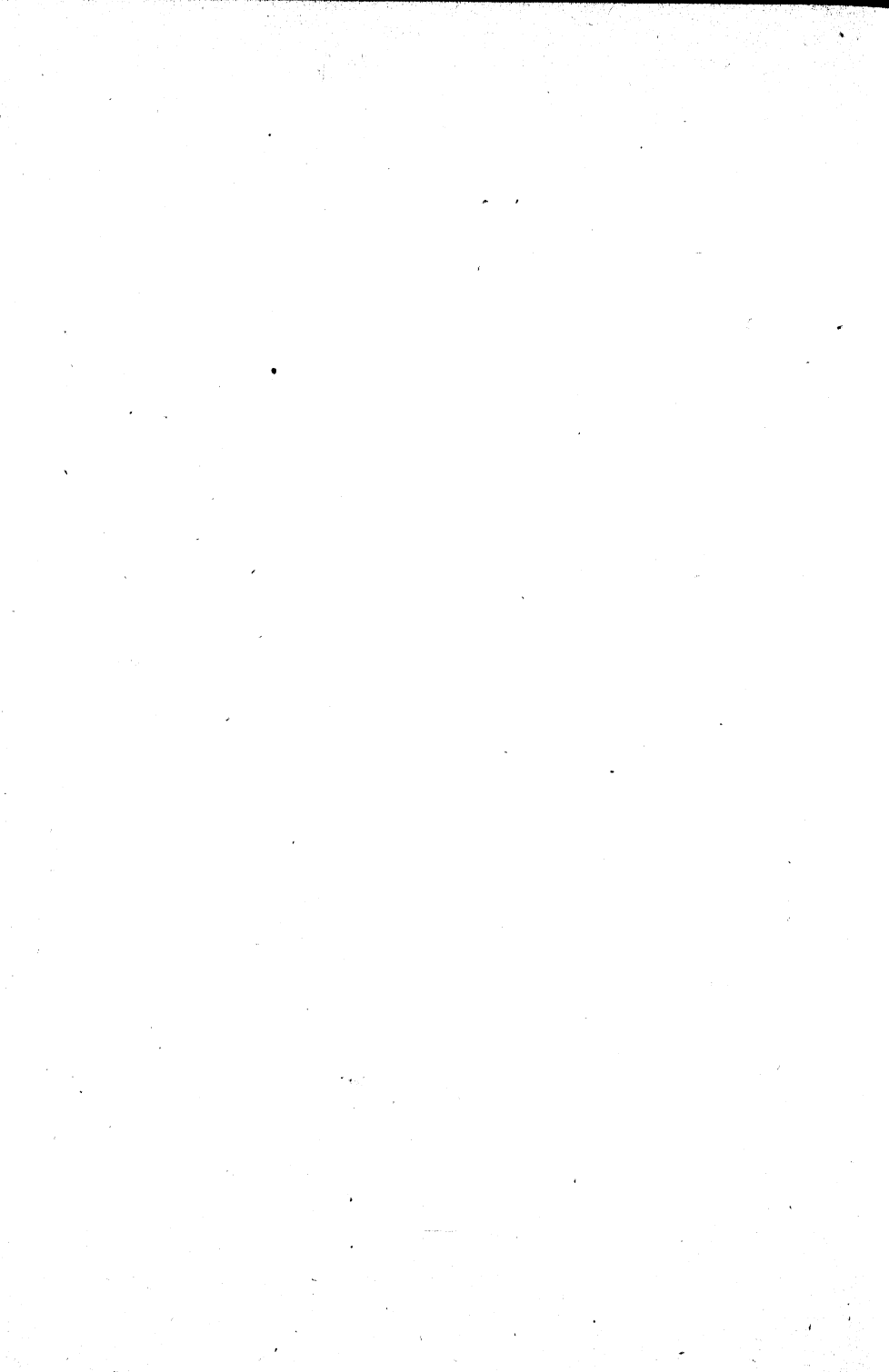
# Ester Wax as a Medium for Embedding Tissue for the Histological Demonstration of Glycogen

BY

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### INTRODUCTION

IT is generally recognized that glycogen is a very labile substance which disappears rapidly from tissues unless treated by special methods of fixation and embedding. The recognized and widely used techniques, advocated by the standard microtomical treatises, call for fixation in a glycogen-precipitating fixative (absolute alcohol; picro-formol-alcohol; picro-dioxane) followed by celloidin embedding.

Since glycogen is considered to be readily soluble in water, if paraffin sections are to be used, special precautions are advocated: sections must be flattened on slides with 70 per cent. alcohol instead of albumen-water; mounted sections, after removal of wax, must be brought into absolute alcohol and then into 1 per cent. celloidin in absolute ether. This latter process covers the section with a thin film of celloidin which 'seems to prevent the diffusion of the glycogen from the section into the water' (Carleton, 1938). Most workers further emphasize that water must be avoided during the subsequent staining processes. On the other hand, Bensley (1939) in a recent account of staining methods for glycogen makes no mention of the necessity for the celloidin film, but merely states that 'tissues may be embedded in paraffin or celloidin'; and when staining 'sections may be brought down to water'.

In recent work on cestode physiology we have had available a considerable quantity of material rich in glycogen, namely, the plerocercoid larvae of the cestode *Ligula intestinalis* which contain seldom less than 30 per cent. glycogen (dry weight; Markov, 1939). It has thus been possible to test the embedding methods on this material on an extensive scale. Confirmatory experiments were carried out with rabbit liver.

### EXPERIMENTAL RESULTS

#### *Ligula intestinalis*

*Paraffin wax.* Pieces of fresh larvae removed from a fish immediately after pithing were fixed in hot (60° C.) picro-formol-alcohol (absolute alcohol saturated with picric acid—90 c.c.; neutral formol—10 c.c.), embedded in paraffin wax, sectioned at $5\mu$, and stained with iodine or Best's

carmine using the modifications of Bensley (1939). Results were briefly as follows:

(i) Some blocks gave sections rich in glycogen, whereas others gave sections poor in glycogen.

(ii) Blocks which gave sections poor in glycogen, and which had only been sectioned about half-way through, when re-embedded in pure wax for long periods (at least overnight) now gave sections rich in glycogen!

(iii) When a 'short-embedded' block, partly sectioned, was immersed in aqueous iodine, the *centre* of the tissue at the block face stained immediately, whereas the periphery stained only after some minutes' immersion.

(iv) Sections from 'long-embedded' blocks or re-embedded blocks, provided they cut properly (which they rarely did), were as rich in glycogen if floated on albumen-water as on 70 per cent. alcohol.

*Ester wax.* Prolonged embedding in paraffin wax had the effect of making material very refractive and sections were usually crumbly and difficult to cut. This latter difficulty was overcome satisfactorily by using the very hard ester wax recently introduced by Steedman (1947). The procedure adopted was as follows:

Fix in picro-formol-alcohol: 2 hrs.
Absolute alcohol (3 changes): 12–24 hrs.
Absolute alcohol/ester wax (1 : 1): 2 hrs.
Pure ester wax: 3 hrs.
Pure ester wax: 12 hrs.—overnight.

Sections were cut with ease at $5\mu$, flattened on albumen-water in the usual way, and stained as before. All sections from blocks embedded in this manner contained great quantities of glycogen filling every available inter-cellular space in the cestode tissue.

In order to test the lability of glycogen, a number of sections were brought down to water, left standing in water, and removed and stained at intervals of one day to determine how rapidly glycogen disappeared from the sections. In contrast to expectations, it was found that *such sections could remain in water for periods of 5–7 days without any appreciable glycogen loss.*

### Liver

The results outlined above—though agreeing with those obtained from similar but much-less-detailed experiments on larval *Diphyllobothrium* (Smyth, 1947)—were so contrary to the accepted views on the lability of glycogen, that it was considered possible that glycogen in such cestode tissue might exist in a form different from that in mammalian tissue, although results of chemical analysis suggests that glycogen in cestodes does not differ significantly from mammalian glycogen (Brand and Oesterlin, 1933; Wardle, 1937; Salisbury and Anderson, 1939).

In view of this possibility, a confirmatory series of experiments was therefore carried out using as a test material pieces of liver from a rabbit previously fed on carrots for 3 days—following the usual practice. Pieces 2–3 mm. thick were fixed in picro-formol-alcohol and embedded in (*a*) paraffin wax, using normal embedding times, i.e. 'short embedding'; (*b*) paraffin wax, using overnight embedding, i.e. 'long embedding'; (*c*) ester wax.

It was found that there was no significant difference between (*a*) and (*b*), but that on the whole sections from these two series were somewhat inferior to ester wax sections as regards the discreteness of the globules and the brightness of the staining. All series of sections could remain standing in water for very considerable periods without glycogen loss. Paraffin wax sections showed some loss after 5 days, but ester wax sections showed not the slightest trace of loss even after 7 days' immersion in water, and it was impossible to distinguish sections that had been soaked in water for this period from those which had been stained without immersion.

## DISCUSSION

The main results in these experiments that require explanation are: (*a*) pieces of *Ligula* require 'long embedding' in paraffin wax in order to retain glycogen, whereas 'short embedding' is sufficient for thin pieces of liver; (*b*) ester wax sections are superior to paraffin wax sections; (*c*) sections of properly embedded material when brought down to water can remain in water for long periods without appreciable glycogen loss.

(*a*) The fact that by prolonged embedding of *Ligula* in paraffin wax the glycogen is held, whereas by short embedding it is not, suggests that with short embedding the wax molecules do not completely permeate into the great amorphous masses of glycogen present in the inter-cellular spaces. This view is substantiated by the fact that when a short-embedded block, partly sectioned, is immersed in iodine the tissue stains instantly in the middle region yet only slowly at the periphery. This we interpret as indicating that the wax, which slows up the movement inwards of the aqueous iodine, has never reached the centre. If the glycogen in such a block was not firmly held by the wax, on sectioning some would fall out as a powder and other masses become loosened to such an extent that they could be lost by mechanical means in later mounting and flattening. This is exactly what happens to sections cut from the block just mentioned; although the face of the block after standing in iodine takes up the iodine intensely—thus showing glycogen to be present—yet sections from this block when stained contain little glycogen in the middle region, i.e. the region where the wax had not properly permeated. If such a block be re-embedded in wax for a long period, it gives sections uniformly rich in glycogen.

It thus seems reasonable to conclude that the glycogen is lost in cutting sections and in the subsequent flattening processes. This must be considered to be purely a *mechanical* effect due to the fact that glycogen is not completely permeated in wax in short-embedded blocks and is thus only loosely held.

If this hypothesis be true, the question immediately arises—why is short embedding sufficient for pieces of liver? This result can be accounted for when the amount of glycogen in liver cells is compared with that present in *Ligula*. In the former, glycogen is present only as small globules which can be penetrated on all sides by wax, whereas in the latter enormous masses of glycogen fill the parenchymal and muscular inter-cellular spaces. It seems self-evident that the time required to permeate the dense masses in *Ligula* will be considerably longer than that required to permeate the small globules in liver cells.

Previous workers have drawn attention to the flattening procedure as being a critical one, as the aqueous albumen would seem a likely place for the generally supposed highly labile glycogen to diffuse out. In one detailed experiment 10 sections of liver were mounted on water, and the same number on 70 per cent. alcohol; it was impossible to distinguish any difference in the amount of glycogen in the slides of the two series. Since this original experiment we have mounted many hundreds of sections flattened on albumen-water without ever getting results suggestive of a loss at this stage, provided material was properly embedded.

(*b*) If good results depend only on getting the paraffin wax right into the glycogen, it is to be expected that long-embedded paraffin wax blocks should give as good results as ester wax. The reason why ester wax gives better results we have already indicated, i.e. prolonged embedding makes tissues exceedingly hard and refractive with the result that sections are frequently difficult to cut and often crumbly, torn, or wrinkled, thus giving glycogen a chance to be lost at a later stage. Ester wax, due to its celloidin-like toughness and strength, permits sectioning of very hard material with great ease and does not introduce the difficulties resultant of paraffin wax embedding. We do not consider, therefore, that *per se* ester wax has any intrinsic property for retaining glycogen, but that like celloidin it merely overcomes, due to its toughness, the technical difficulties introduced by alternative methods.

(*c*) It has long been accepted that glycogen in sections is a highly labile substance, and for that reason most previous techniques have emphasized that sections should be handled without taking them below 60 per cent. alcohol—the strength at which alcohol precipitates glycogen from aqueous solutions. When, for example, aqueous reagents—or reagents with a low alcohol content—were used in the staining technique (i.e. as in counterstaining in the Best carmine method), the celloidin film technique was introduced to prevent diffusion of the labile molecules into the surrounding medium.

Basing our hypothesis on the above results, we believe that—providing material has been properly fixed, thoroughly dehydrated, cleared and embedded, sections not damaged in cutting, and smoothly flattened—when wax is removed and slides brought down to water, no glycogen is lost by dissolving in water. The fact that slides can be stored in water for 7 days and still be packed with glycogen must force us to revise prevalent ideas concerning the

lability of glycogen, and points to the inevitable conclusion that glycogen is either in an insoluble form at this stage or is 'held' in some way.

It is at once apparent that the answer to this problem lies in a greater knowledge of the structure and properties of glycogen. Recent work has shown that glycogen is a mixture of two complex polymers with highly branched molecules: *lyo*-glycogen, whose molecule is weakly linked to proteins and is *soluble*, and *desmo*-glycogen, whose molecule is strongly linked to proteins and is *insoluble* (Myer, 1942; Carter and Record, 1939). Since liver-glycogen contains only about 15 per cent. of the *desmo*- form (Genkin, 1946) it cannot possibly account for the amount present in our sections; we resort therefore to the alternative conclusion that the *lyo*- form has at least been partly—if not entirely—retained. We believe that the *lyo*- form does not diffuse from sections immersed in water because its large branched molecules are held by the associated protein network which has been precipitated around them. Lison (1936) has already put forward this hypothesis in more general terms, and, as he has pointed out, it adequately explains why a protein-coagulating fixative such as picric acid—while not precipitating glycogen *in vitro*—is an excellent fixative for glycogen in tissues.

## Summary

1. The effect of paraffin wax embedding on the lability of glycogen in sections has been tested using as material (*a*) plerocercoid larvae of *Ligula intestinalis*; (*b*) rabbit liver.

2. It was found that glycogen in tissues was very impermeable to wax, and where large masses of glycogen occur prolonged embedding is essential.

3. Improperly embedded material lost glycogen easily; it was concluded that this loss takes place during the processes of cutting and flattening sections.

4. The effect of prolonged embedding was to make tissue hard and refractive. This difficulty was overcome by embedding in Steedman's ester wax.

5. Ester wax blocks allowed thin sections of very hard material to be cut with ease.

6. Ester wax sections brought down to water did not lose glycogen even after standing in water for 7 days.

7. It is emphasized that glycogen exists in two forms: an insoluble *desmo*-form and a soluble *lyo*- form. It is suggested that the latter does not dissolve from sections in water because its highly branched molecule is held by the coagulated protein network.

## REFERENCES

BENSLEY, C., 1939. Stain Tech., **14,** 47.

BRAND, TH. VON, and OESTERLIN, M., 1933. Z. vergl. Physiol., **20, 251.**

CARLETON, H. M., 1938. *Histological Technique.* Oxford (Univ. Press).

CARTER, S. R., and RECORD, B. D., 1939. J. Chem. Soc., **1,** 644.

GENKIN, A. M., 1946. Biokhimiya, **11,** 155 (abstract only).

LISON, L., 1936. *Histochimie Animale.* Paris (Gauthier-Villars).

MARKOV, G. S., 1939. C. R. Acad. Sci. U.R.S.S., **25,** 93.

MYER, K. H., 1942. *Natural and Synthetic High Polymers.* New York (Interscience Publishing Inc.).

SALISBURY, L. E., and ANDERSON, R. J., 1939. J. Biol. Chem., **12, 505.**

SMYTH, J. D., 1947. J. exp. Biol., **24,** 374.

STEEDMAN, H. F., 1947. Quart. J. micr. Sci., **88, 123.**

WARDLE, R. A., 1937. Canad. J. Res., **15, 115.**

# A Simple Method for Orientating Small Objects for Sectioning, with Special Regard to Nematodes

BY

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**With one Text-figure**

FOR solving certain anatomical problems in free-living nematodes serial sections are necessary. Various methods of orientation proved unsatisfactory for these small objects (in this case $700\mu$ in length and $30\mu$ in width). The following method was worked out and found excellent:

In a drop of water the nematode is heat-paralysed, stretched at 50–60° C., and pipetted into Bouin's mixture (Graupner). After 24 hours it is transferred to 30 per cent. alcohol (4 hours), 50 per cent. alcohol (3 hours), a mixture of 50 per cent. alcohol and aniline (1 : 3) (1 hour), pure aniline (1 hour), bergamot oil (3 hours), paraffin wax I (3 hours), paraffin wax II (1 hour), in which it is embedded.
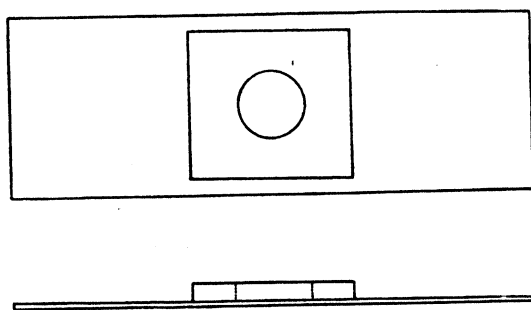


TEXT-FIG. 1

All the manipulations except the last one are performed in the usual way and practically no shrinkage takes place.

The difficulties in orientation preceding the embedding are overcome in the following way:

A square glass plate 2 mm. thick and with side of 22 mm., provided with a central circular hole of diameter 15 mm., is fixed by means of Canada balsam to a microscope-slide (see Text-fig. 1). When the Canada balsam has dried the hole is filled with the paraffin wax II, into which the object is transferred from paraffin wax I for orientation and embedding. By means of a heated needle the object is placed 1 mm. above the bottom in the paraffin

wax, which as far as possible is kept in a liquid state by cautious heating over a small flame. These manipulations are performed under a binocular microscope at about 40-fold magnification. Under these circumstances it is easy to orientate the object in such a way that the intended plane of section is exactly parallel with the bottom. When the object has been placed in position, the slide is immediately plunged into water of 10–12° C. (If the water has a higher temperature, the needles of solid paraffin will have the same order of size as the object, and a very loose and unsatisfactory embedding results.)

When the paraffin wax has become solid a block with sides about 5 mm. long is cut out enclosing the object.

A block of wood with a block of paraffin wax fused on it is placed in the microtome and sections are cut until a convenient area of its surface is plane.

By means of a forceps the object block with the plane lower surface downwards is held 5 mm. above the block in the microtome. A very hot lancet is placed in the interspace between the two blocks (without touching either of them). When the radiant heat has melted a fine film of paraffin wax on the two surfaces, the blocks are rapidly fused to each other. After cooling, the block-holder of the microtome is lowered 2 mm. by means of the screw and sectioning may take place. If the radiant heat has been applied accurately, the plane of section will be quite parallel to the plane lower surface of the block containing the object.

Thus an exact orientation (at magnification ×40) is obtained and maintained during sectioning in a purely mechanical way without the drawbacks of a subjective estimation, which is practically impossible in the case of these small objects even if they have been stained beforehand.

By employing this method it has been possible to obtain exactly longitudinal sections in a large number of these small objects, which cannot be seen with the naked eye.

Exactly the same technique has been satisfactorily employed for the sectioning of Protura and Mallophaga—without the use of diaphanol.

# A New Single-control Micromanipulator

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**With one Plate**

### Introduction

SEVERAL types of micromanipulator have been described (for reviews see Peterfi, 1928; Chambers and Kopac, 1937; Seifriz, 1936). The growing importance of micrurgical techniques in bacteriology, electrophysiology, embryology, plant physiology, and experimental cytology have focused attention on the problem of micromanipulator design. The question has assumed a new importance with the recent development of two revolutionary advances in microscopy. The first of these is the high-performance reflecting microscope of Dr. C. R. Burch (see Burch, 1947; Barer, 1948a). The instrument in use at Oxford has a working distance of 13 mm. when used at N.A. 0·65. This long working distance is exceptionally useful for microdissection, as it enables the usual type of moist chamber and hanging drop preparation to be dispensed with. It is now also possible to perform micromanipulation on the surfaces of intact organs *in situ*, e.g. the liver, spleen, kidney, or brain of anaesthetized animals (Barer, unpublished results). The second outstanding advance in microscopy has been Zernike's phase-contrast method (Zernike, 1942; Burch and Stock, 1942; Barer, 1947b, 1948b). This enables living unstained cells to be studied under optical conditions vastly superior to those hitherto available. There is no doubt that much early work will now have to be repeated and extended by the use of this method. The technique of microdissection by phase-contrast illumination is far from easy, but the results amply repay the trouble.

### Features Desirable in a Micromanipulator

#### 1. *Single Control*

Most micromanipulators, e.g. those of Chambers and Peterfi, depend on three main controls, one for each direction in space. These usually take the form of screws which operate rack-and-pinion mechanisms or cause the spreading-apart or sliding motion of metal plates. While such methods of control may give very precise linear movements, the latter are rather limited

and unnatural, since in order to go from point A to point B, one can only travel via X, Y, and Z. This would not be a very convenient way of writing one's name, nor is it the ideal method for microdissection. The ideal micro-manipulator should enable the operator to move an instrument smoothly and rapidly along any desired path between two points in space. So far as we are aware this ideal condition has yet to be achieved, and the mechanical problems involved are very formidable. Fortunately an approach can be made to the problem if we are prepared to sacrifice complete smoothness of control in three dimensions, substituting instead smooth movement in one plane combined with an independent movement in a direction perpendicular to this plane. Simple micromanipulators based on this principle have been described by Buchthal and Persson (1936) and by Schuster (Barer, 1947a). These instruments allow any desired movement in a vertico-lateral plane, with a screw control for antero-posterior movements. Their great advantage is that all movements are operated from a single control, which remains in the hand during the entire operation, but their inherent weakness lies in the fact that for most high-power work the greatest precision of movement is required in the *horizontal* plane, i.e. the plane in focus under the microscope. Thus, although such micromanipulators are quite satisfactory for use at medium magnifications (up to about 500 times), their use at higher powers demands considerable skill. Of the instruments which allow complete freedom of movement in a horizontal plane perhaps the best known is that of de Fonbrune (1932, 1937). This outstanding high-precision design has been developed over a period of many years and the present model must rank as one of the finest available. It is unusual in that it works on pneumatic principles. Air pressure from three mutually perpendicular pistons is transmitted through three rubber tubes, on to three tambours, resembling aneroid barometers, which are connected to a lever holding the micro-instrument. The pressure in the pistons is controlled by a single handle. Movement of the handle in a horizontal plane moves one or both of the horizontal pistons, and vertical movement is obtained by a screwing motion of the handle. With practice something very near to complete freedom of movement in three dimensions can be achieved. The de Fonbrune micromanipulator incorporates a number of other important features which will be referred to below.

## 2. Freedom from Vibration

The elimination of vibration is essential in micrurgy. Factors which may help in this respect are (1) massive construction; (2) clamping the micro-manipulator and microscope to a common base-plate; (3) remote control. Most commercial micromanipulators rely on factors (1) and (2), either alone or together. Massive construction is usually no disadvantage in high-power cytological work, but it may be an encumbrance if the instrument is to be used for other types of work, where it may require to be poised in mid-air at an angle. The same may sometimes be said of factor (2). Some micro-manipulators are only designed for use for one specific purpose and a more

or less fixed base-plate assembly is provided. This greatly limits the versatility of the instrument and in general it is preferable to have an instrument which can be made quite independent of the microscope if required. A common base-plate with clamps can always be added as an accessory. The Schuster micromanipulator is unusually adaptable in this respect. Although usually mounted on a heavy base, the effective part of the instrument can be detached and mounted in any position or orientation on a clamp or sliding bar. This makes it particularly useful for accurate positioning of electrodes. Remote control, i.e. absence of rigid connexion between the control screws or handle and the micro-instrument holder itself, is obviously a valuable feature in reducing vibration, and accidental jarring of the controls may not be transmitted to the micro-instrument itself. Although remote control was introduced into a form of the Chambers micromanipulator, the de Fonbrune is the remote-control instrument *par excellence*. Here the single-control handle and the instrument proper are built as two independent units, connected only by a considerable length of flexible rubber tubing. The instrument holder is comparatively light and delicate but freedom from vibration and accidental jolts is assured by this independence, which also enables the instrument to be used to some extent for other than cytological work. For maximum stability and robustness there is no doubt that massive construction is an advantage. On the other hand, remote control is often very convenient for ease of manipulation and may enable a lighter type of construction to be adopted.

### 3. *Freedom from Play (backlash or lost motion)*

It is essential that the instrument holder should respond without delay to any movement of the controls, and that there should not be any further movement or 'creep' on sudden removal of the hand. This 'dead-beat' condition can only be achieved by careful attention to details of design. If differential screw feeds or rack-and-pinion movements are used they must be made with a considerable degree of precision. Sliding surfaces and bearing points must be of suitable material and should be so designed that the effects of wear are eliminated. Multiple levers and cam mechanisms are on the whole to be avoided as they are rarely free from play. This fault makes the design of micromanipulators on the usual principles of a pantograph rather impracticable.

### 4. *Variable Sensitivity of Control*

It is extremely useful to be able to vary the sensitivity of control according to the magnification of the microscope. If this cannot be done it may be found that work under low powers is too slow, especially with screw controls, as it may take a long time to traverse the field of a low-power objective. Again with screw controls a large field can as a rule only be traversed by a series of turns of the screw, thus leading to intermittence and jerkiness of operation. Some degree of variation of sensitivity can be introduced into the Chambers and rack-and-pinion instruments by attaching special levers on to

the operating screws, but this profusion of rods jutting out in various directions of space is clumsy and inconvenient. One of the most successful methods of achieving smooth and *continuous* variability of sensitivity is that adopted by de Fonbrune. A sliding collar is fitted around the vertical control handle. Rods run from this collar to each of the two pistons which control movements in a horizontal plane. A movement of the sliding collar alters the angle of movement of these piston levers and thus the range and sensitivity of motion in the horizontal plane. The vertical movement is unaffected, but variation of this is relatively unimportant for most work. This mechanism is exceedingly valuable, but three disadvantages should be noted. In the first place the transition from rather coarse to very fine movement is not linear but tends to be rather abrupt—most of the reduction takes place in the final few millimetres of travel of the sliding collar. This is not a very serious fault since the variation is continuous. Secondly, the range of movement diminishes with increasing sensitivity; for example, if the sensitivity is doubled the range of movement is halved. This is inconvenient if very fine work has to be done over a very large field, although this requirement is rare in practice. In principle a screw mechanism could be made free from this fault. The third disadvantage is rather more serious. A movement of the sliding collar not only alters the angle of movement of the horizontal piston levers but moves the pistons bodily by a small amount, thus shifting the micro-instrument. The latter thus has to be recentred and may even move out of the field of view altogether.

### 5. *Limitation of Movement to the Field of View*

In most micromanipulators no provision is made for this. Thus if by chance the micro-instrument should wander out of the field of view it may be very difficult to find it again. This is particularly so when working at high magnifications, when the only practicable plan may be to return to an objective of lower power. In the de Fonbrune instrument the movement of the control handle is limited by a metal circle. The range of movement can be made to correspond exactly with the field of view by adjustment of the sliding collar mentioned above.

### 6. *Rapid Centration*

Some form of coarse movement to enable rapid centration of the micro-instrument in the field of view is highly desirable. This may not be so important in instruments made to clamp in a fixed position relative to the microscope, and when micro-instruments of a standard length are used, but it is almost essential in other cases. A coarse vertical motion is also desirable.

### 7. *Robustness, Price, &c.*

This is to some extent connected with the question of massive construction. In choosing between two instruments of roughly equal performance it is natural to decide in favour of the more robust and less easily damaged. It is

also important that any accidental damage should be capable of easy repair, preferably by any competent workshop technician, and that spare parts should be available at low cost. This puts delicate instruments and those involving high-class precision engineering at a disadvantage. It is often possible to cut down the price of an instrument very considerably by employing methods of design not involving high-precision fits. Price and robustness are important considerations in a micromanipulator, since for most work a pair of instruments is required.

### THE NEW MICROMANIPULATOR

Although the resemblance is at first difficult to find, the present instrument began as a simple modification of the Schuster micromanipulator. As we have seen, the chief drawback of the latter is the fact that smoothest motion occurs in a vertical plane, whereas we should like it in the horizontal plane. A fairly obvious modification was thus to turn the instrument through 90°, with the control handle vertical, and to attach the micro-instrument holder to the upper sliding plane. The first rough models gave surprising results. The action in a horizontal plane was beautifully smooth and controllable, and by attaching the control handle to a heavy block of metal which could slide on a polished surface it was possible to trace most complicated patterns with extreme rapidity. Unfortunately, in order to obtain perfect uniplanar motion the vertical movement had been sacrificed. Experiments were made with a simple tilting vertical motion by means of a screw fixed through the base of the instrument, but this did not prove sufficiently good for high-power work. At the same time the first results seemed so promising that it was felt justifiable to build a much more ambitious model incorporating several of the desirable features described above.

The construction of the latest version of the new instrument will be seen from the diagram (Text-fig. 1) and photograph (Pl. I). The steel ball-bearings (2) of the horizontal movable brass plate (1) rest in a groove and a flat (18) on the horizontal fixed brass plate (6), clamped by the adjustable spring (3). The double-ball-ended lever (4, 5, 7) locates in two cones in the plates (1) and (6) and is gripped by three small steel balls (9) mounted on a slider (10) which moves on three rods (11) fixed to the plastic handle (8) and the coned base (17). Various types of bearing points, including jewel tips, were tried at (9), but the steel balls were found to be most satisfactory and did not score the lever (7).

The coned base (17) is clamped to the screw-mounted steel ball (12), the lower end of the screw making contact with a steel ball (27) fixed to the lever (30), which moves about the ball fulcrum (26). The balls (20, 20), fixed to the vertical movable plate (23), rest in V-grooves and a flat (19, 19) of the vertical fixed plate (22). These two plates are held together by the adjustable spring (31). The lower end of the vertical plate (23) is fixed to a ball (32) resting on the lever (30). The balls (24, 24) of the heavy base (33) rest in grooves (25) and flats on the undercarriage (34). The base (33) and undercarriage (34)

can be fixed together by the clamp (14). The short double-ball-ended lever (15) locates in a hole (16) in the base (33) and in a cone (28) in the under-carriage (34).

The micro-instrument holder (35) is clamped to the tool post (29) and is adjustable vertically, horizontally, and angularly before locking.
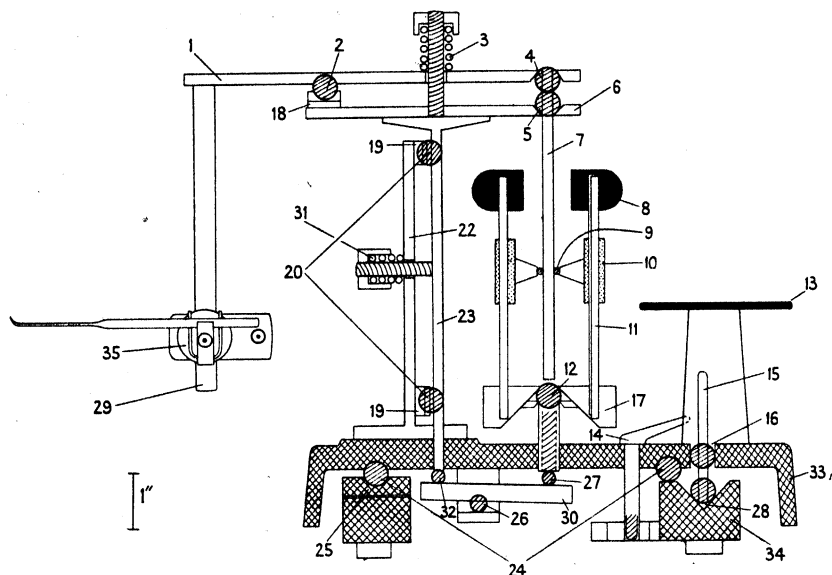


TEXT-FIG. 1. Diagram of micromanipulator.   For explanation of the numbers see text.

## OPERATION

The micromanipulator is placed at a convenient distance from the micro-scope, and if desired it can be clamped to a common base. (The three sup-ports on the undercarriage can be conveniently sunk in three slots.) The micro-instrument holder is adjusted to a suitable height and the micro-instrument inserted. It should be noted that the type of holder we have found best is a small version of the Singer clamp (Registered Design No. 847073). This holder will accommodate anything from a very fine needle to a tube 9 mm. in diameter, thus allowing the use of non-polarizable micro-electrodes of wide bore. The micro-instrument is held in place by pressure from a metal leaf against two V slots. The micro-instrument does not have to be held horizontally as is the case with many other types of holders, but can be used at any desired angle. The desirability of having a rack-and-pinion movement of the holder on the post (29) was considered, but it was felt to be an unnecessary refinement for most work.

The clamp (14) is now loosened and the tip of the micro-instrument is centred rapidly in the field of view by means of the handle (15) which causes the base (33) to slide on the undercarriage (34). This movement of the base

on the undercarriage is of wide range and can even be used for very low-power micromanipulation. As soon as the micro-instrument is centred the clamp (14) is tightened, preventing any further movement of the base.

The hand of the operator rests on the plastic hand-rest (13). The fingers grasp the handle (8). Movement of the handle produces reduced motion through the balls (9) working on the ball (12) to the lever (7). This moves the plate (1) to which the tool post (29) is fixed. The plate (1) can only move in a horizontal plane. Thus a rocking movement of the handle (8) enables any desired curve to be traced in a horizontal plane, at any desired speed. Twisting of the handle (8) rotates the screw-mounted ball (12). This moves the lever (30) about its ball fulcrum (26), and allows the plate (23) to be raised or lowered against the fixed plate (22). The two horizontal plates (1) and (6) and with them the tool post (29) are thus moved vertically.

The slider (10) enables the sensitivity of the instrument to be varied through a wide range. When at its highest position, close to the handle (8), a fairly coarse movement of wide range is obtained, suitable for low-power work. In the lowest position, close to the coned base (17), the movement is very delicate, but reduced in range. At the same time it will be noted that the movement of the handle is automatically limited by its circumference coming into contact with the lever (7). This restricts the range of movement of the micro-instrument tip. In practice it will be found most convenient to move the slider (10) until a position is found at which the range of movement of the micro-instrument tip corresponds with, and is limited to, the field of view of the microscope in use at the time. The sensitivity of the instrument can be altered at will without any undesirable movement of the micro-instrument.

### REMARKS

We are now in a position to consider how far the present instrument meets the requirements (1) to (7) discussed above.

1. Although we have not achieved perfect control in three dimensions we have virtually perfect control in the two dimensions of a horizontal plane, combined with vertical movement controlled by the *same single handle.* The majority of cytological work can be carried out in a horizontal plane with only occasional use of the vertical movement. One minor fault must be pointed out here. It would be desirable to be able to carry out a vertical movement without risk of a slight horizontal displacement. This is sensibly so at low and medium sensitivities. At very high sensitivities, however, when the slide (10) is close to the coned base (17), the effective leverage is so great that the horizontal movements are carried out against appreciably less resistance, making it more difficult to twist the handle (8) without producing some slight horizontal displacement, though this can be done with practice. However, this tendency can be reduced to a large extent by tightening the screw spring (3), which increases the resistance to horizontal movement. The tension of the same spring can be adjusted for maximum ease of manipulation at any sensitivity.

2. The instrument will be found to be remarkably free from vibration. Even if the handle (8) is struck against the lever (7) with considerable force the vibration of the micro-instrument is relatively slight and rapidly damped. Movements of the hand on the hand-rest are usually without obvious effect. This feature has been achieved largely by massive construction.

3. Despite numerous trials no appreciable play has been noticed. This very important result has been achieved entirely without any fine-limit precision-engineering methods, but mainly by attention to the design of the bearing surfaces. Any tendency to the development of play as a result of wear can be taken up by adjustment of the tensions of the two springs (3) and (31). The design is such that the bearing surfaces would merely 'bed' into one another as wear occurs.

4. Sensitivity can be varied quickly and easily over a wide range, and without moving the micro-instrument. The variability is continuous and sensibly linear, with no sudden transition from coarse to fine movement. It will be noted, however, that the range is automatically reduced as the sensitivity is increased.

5. The range of movement can be limited at will to the field of view.

6. Rapid centring is achieved by movement of the base (33) on the under-carriage (34).

7. The instrument is exceptionally robust. There are no delicate components whatsoever, and no high-precision work is involved. Should repairs ever be necessary they can be carried out by any competent workshop technician. It may perhaps be stated that the instrument has been subjected to considerable mechanical violence on a number of occasions in order to test its strength, and on no occasion has damage ensued. The same robustness is evident in the very versatile micro-instrument holder. The lack of any high-precision work enables the instrument to be made relatively cheaply, bringing a *pair* of micromanipulators within the reach of most investigators. The possibility that the instrument might be used at marine biological stations has been considered, in deciding the best type of corrosion-resisting finish. Corrosion of metals by sea-water is a very difficult problem, but it is felt that chromium plating combined with reasonable care offers the most practical solution.

In conclusion it may be mentioned that the instrument has been tried out for a number of purposes, including microdissection by phase-contrast illumination, and has proved very successful in every way.
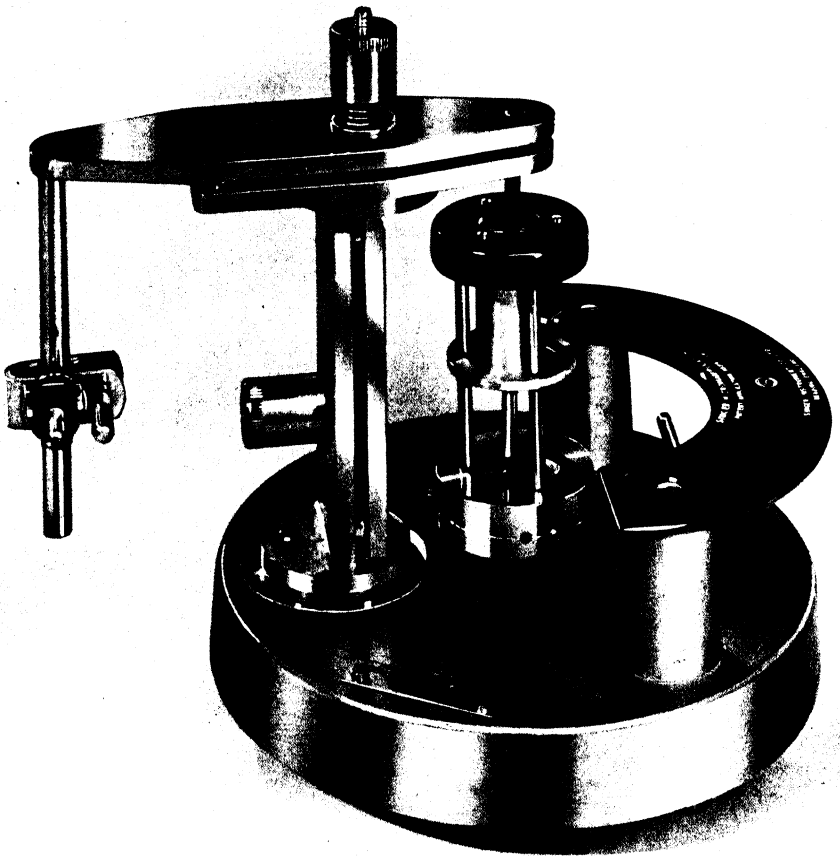
R. BARER AND A. E. SAUNDERS SINGER—PLATE I

## SUMMARY

New developments in microscopy and electrophysiology have brought a renewal of interest in methods of micromanipulation.

A number of features desirable in the design of a micromanipulator is discussed.

A new micromanipulator is described. Its outstanding features are (1) single control; (2) massive construction with freedom from vibration; (3) freedom from play; (4) continuous variability of sensitivity; (5) limitation of range of movement to the field of view; (6) rapid low-power centring; (7) unusual robustness combined with delicacy of movement achieved without any high-precision methods. The performance of the instrument is adequate for high-power cytological work and for microdissection by phase-contrast illumination.

## REFERENCES

BARER, R., 1947*a*. J. Anat. Lond., **81**, 259. (Appendix by E. H. J. Schuster.)
—— 1947*b*. Quart. J. micr. Sci., **88**, 491.
—— 1948*a*. British Science News, **1**, no. 6, 12.
—— 1948*b*. Ibid., **1**, no. 9, 10.
BUCHTHAL, F., and PERSSON, C., 1936. J. sci. Instr., **13**, 20.
BURCH, C. R., 1947. Proc. Phys. Soc., **59**, 41.
—— and STOCK, J. P. P., 1942. J. sci. Instr., **19**, 71.
CHAMBERS, R., 1924, in Cowdry's *General Cytology*, Sect. V. Chicago.
—— KOPAC, M. J., 1937, in McClung's *Microscopical Technique*. Oxford University Press.
DE FONBRUNE, P., 1932. C. R. Acad. Sci., Séance Oct. 10.
—— 1937. *Micromanipulateur Pneumatique et Micro-forge.* Paris (Société Industrielle d'Imprimerie).
PETERFI, T., 1928, in Abderholden's *Handbuch der biologischen Arbeitsmethoden*, Abt. 5, Teil 2, Heft 5, 479.
SEIFRIZ, W., 1936. *Protoplasm*, New York (McGraw-Hill).
ZERNIKE, F., 1942. Physica, **9**, 686, 974.

# SINGER MICROMANIPULATOR

(patent application 11559/48)
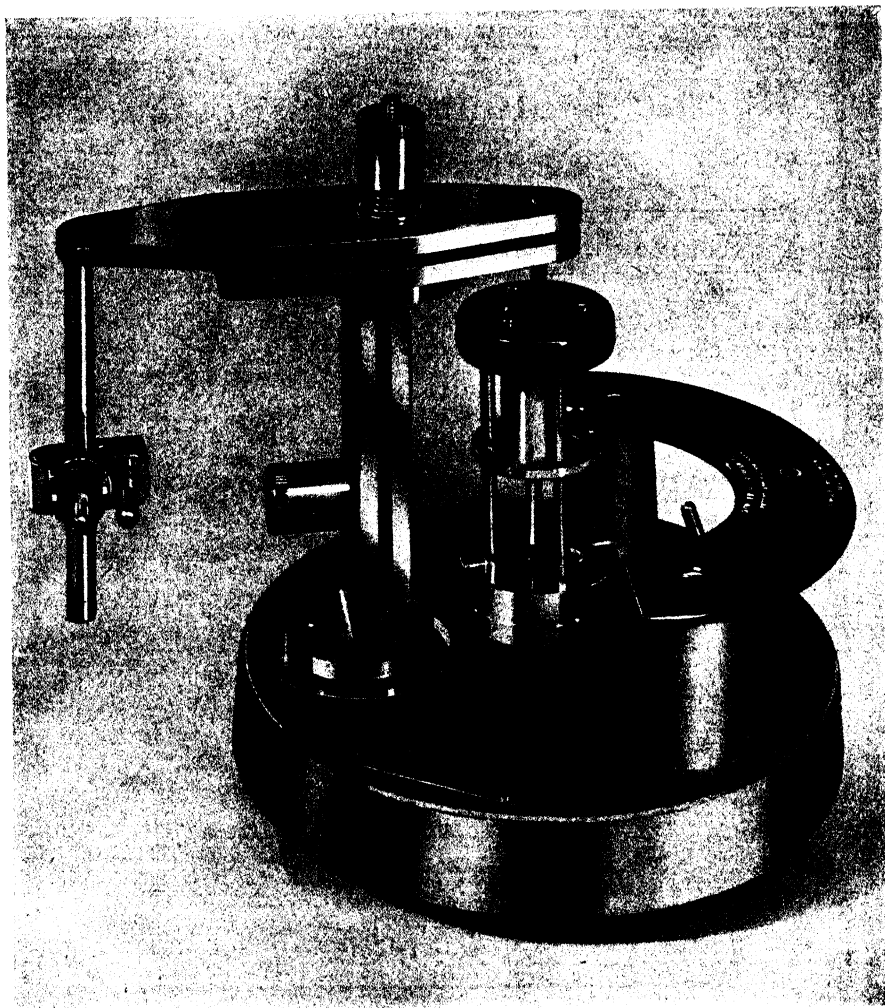


**Designed by the Singer Instrument Company Limited in collaboration with Dr. Robert Barer of the Department of Human Anatomy, University of Oxford**

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